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Praziquantel-resistance in *Schistosoma mansoni:* role of efflux pumps, phenotypic characteristics and proteomics analysis

Tese de Candidatura ao grau de Doutor em Biologia Básica e Aplicada submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Publications

The following articles were published or submitted to publication in the ambit of this thesis, having been the basis of its drafting:

Pinto-Almeida, A., Mendes, T., Armada, A., Belo, S., Carrilho, E., Viveiros, M., and Afonso, A. 2015. The role of efflux pumps in *Schistosoma mansoni* Praziquantel resistant phenotype. *PLoS ONE*. 10(10):e0140147. doi:10.1371/journal.pone.0140147.

Pinto-Almeida, A., Mendes, T., de Oliveira, R.N., Corrêa, S.A.P., Allegretti, S.M., Belo, S., Tomás, A., Anibal, F.F., Carrilho, E., and Afonso, A. 2016. Morphological characteristics of *Schistosoma mansoni* PZQ-resistant and -susceptible strains are different in presence of Praziquantel. *Front Microbiol*. 7:594. doi: 10.3389/fmicb.2016.00594*.*

Pinto-Almeida, A., Mendes, T., Ferreira, P., Belo, S., Anibal, F.F., Allegretti, S.M., Carrilho, E., and Afonso, A. Comparative proteomics reveals characteristic proteins on Praziquantel-resistance in *Schistosoma mansoni*. *Submitted manuscript.*

Financial support

This work was supported by “Fundação para a Ciência e a Tecnologia” (FCT), from Portugal, through a PhD fellowship (SFRH/BD/51697/2011) and by the project PEst-OE/SAU/UI0074/2014. By “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq n° 400168/2013-8 and CNPq n° 375781/2013-7), and “Fundação de Amparo à Pesquisa do Estado de São Paulo” (FAPESP nº 2009/54040-8, FAPESP nº 2009/16598-7, and FAPESP nº 2008/04050-4), from Brazil.

Acknowledgements

This work was only possible due to the collaboration of all the people that were with me during this period of growth and maturation. I express my gratitude to all of them and in a particular way to my mentors, Dr. Ana Afonso and Professor Ana Tomás.

A special acknowledge to Dr. Ana Afonso, for accepting to be my supervisor, and always being by my side, helping me and supporting me all the times. Thank you for make me grow as a professional and as a person.

I have to thank my PhD program – GABBA, in particular the 15th edition team with whom I shared some of the best moments of my life. A special thanks to Professor Ana Tomás for the co-supervision, to Catarina Carona for all the help with bureaucracy, and to all the GABBA members for giving me this wonderful opportunity.

To the Medical Parasitology Unit, Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa for accepting to be my laboratory host in Lisbon, especially to Professor Silvana Belo, and all the members of Medical Helminthology and Malacology group, for all the support.

To the Medical Microbiology Unit, Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, specially Professor Miguel Viveiros and Dr. Ana Armada, for helping me with the efflux pumps assay.

To the São Carlos Institute of Chemistry, Universidade de São Paulo for accepting to be my laboratory host in Brazil, especially to Professor Emanuel Carrilho, and all the members of BioMics group, for all the support.

To the Institute of Biology, Universidade de Campinas, in Brazil, for their support with the scanning electron microscopy, especially to Professor Silmara Allegretti, Dr. Rosimeire de Oliveira, and Dr. Sheila Corrêa.

To the Laboratory of Parasitology, Departamento de Morfologia e Patologia, Universidade Federal de São Carlos, in Brazil, especially to Professor Fernanda Anibal, and all the members for their support.

To the Genomics and Computational Biology Group Oswaldo Cruz Foundation, FIOCRUZ, Minas Gerais, Brazil, especially Professor Guilherme Oliveira, Dr. Ângela Volpini, Dr. Flávio Araújo and Dr. Fabiano Pais, for all the support.

To the Mass Spectrometry Laboratory at Brazilian Biosciences National Laboratory, CNPEM, Campinas, Brazil, especially to Dr. Bianca Alves Pauletti, for their support with the mass spectrometry analysis.

A special thanks to Dr. Tiago Mendes, who has always been by my side throughout this hard work, as a colleague and a friend.

To Dr. Silvia Ferreira, Dr. Ricardo Oliveira and Dr. Giovana Leite, for helping me with the protein extraction, preparation and electrophoresis. To Dr. Juliana Alberice for helping me with mass spectrometer.

To my family, especially, my mom and my uncles Maria Antónia and António Ramiro, for their love and attention during these journey, and for have always been by my side with understanding and patience.

To my love “Nha Cretcheu”, my partner, for everything especially for her unconditional support, and to my sweet child Hugo, which together allowed me to know a new concept of Love. Thank you for making my life a better experience!

Finally, thanks to all the people that I did not mention, but that somehow have been there during this journey. Thanks to all!

À minha querida mãe e à memória do meu amado pai, que com certeza me guiará para sempre.

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Summary

Schistosoma mansoni is one of the causative agents of schistosomiasis, which is a neglected tropical disease with great importance to public health in Africa, Asia and South America. It is estimated that about 249 million people are infected with this disease and another 732 million are at risk of infection. Praziquantel is the only available drug to treat schistosomiasis, and several mass-treatments have been performed with the same drug. However, in the last years, the number of reported cases of resistance to Praziquantel has been increasing and there is concern about the future of the schistosomiasis treatment. Therefore, it is very important to study the phenomenon of resistance to uncover the genetic mechanisms involved and its associated characteristics. Nevertheless, since the resistant strains previously studied were obtained from patients, it is not possible to guarantee that certain characteristics are, in fact, related to drug resistance, due to lack of an isogenic strain for comparison. Given this limitation, within the ambit of this project, it was developed a Praziquantel-resistant strain of *S. mansoni*, which is isogenic to its susceptible parental strain, except for the resistance determinants.

Efflux pumps are often indicated as being involved in chemotherapy failures by other drugs. Therefore, in this study, we tested the hypothesis of the involvement of efflux pumps in Praziquantel-resistant phenotype in *S. mansoni*. For that, an Ethidium Bromide accumulation assay was performed in the presence of an efflux pumps inhibitor (Verapamil), as well as a susceptibility assay with several Praziquantel concentrations in the presence or absence of the same inhibitor, and a qRT-PCR to determine the expression levels of the gene *SmMDR2*. Comparing the results obtained for each strain, we found that resistant males have a higher efflux pump activity, as well as a lower susceptibility to the drug and an increased expression of the target gene.

In this thesis, the presence of morphological differences between the two strains was also assessed. With the use of scanning electron microscopy, males and females from both susceptible and resistant strains were examined in the presence and absence of Praziquantel. We found significant tegumental damages in the susceptible strain exposed to the drug, but not in the resistant strain. Moreover, optical microscopy of the eggs demonstrated that those released by resistant strain worms are significantly

smaller than those of susceptible strain. The use of mass spectrometry allowed the determination of the proteome of males and females from both strains under drug exposure or not. This permitted to characterize the proteome of each strain and to identify 60 different proteins, some of which differentially expressed between the different groups.

Thus, taking advantage of the development of a new model for the study of Praziquantel-resistance in *S. mansoni*, it was possible to determine some differences between the two strains, which possibly are associated with Praziquantel-resistance in *S. mansoni*. Namely, this study strongly suggests the involvement of efflux pumps in Praziquantel-resistance in *S. mansoni*, and more research is needed to assess the possibility of applying combined therapies with efflux pumps inhibitors to increase worm susceptibility to the Praziquantel-treatment. In addition, the morphological changes observed are a very important finding, because these changes may be occur *in vivo* and have impact in the resistant parasite biology, and consequently on disease's transmission and pathology, namely in a neuroschistosomiasis scenario. Moreover, the elucidation of the proteome of both strains will be important for future works, representing a significant progress in the study of Praziquantel-resistance in *S. mansoni*.

Sumário

Schistosoma mansoni é um dos agentes causadores de schistosomose, uma doença tropical negligenciada com grande importância para a saúde pública em África, Ásia e na América do Sul. Estima-se que cerca de 249 milhões de pessoas se encontrem infetadas com esta doença e outros 732 milhões estejam em risco de infeção. Praziquantel é o fármaco disponível para o tratamento da schistosomose, e vários têm sido os tratamentos em massa realizados com este mesmo medicamento. Porém, nos últimos anos, o número de casos reportados de resistência ao Praziquantel tem vindo a aumentar, existindo uma grande preocupação sobre o futuro do tratamento da schistosomose. Por isso mesmo, é fundamental estudar este fenómeno de resistência, tentando descobrir os mecanismos genéticos envolvidos e as características associadas. Contudo, uma vez que as estirpes resistentes que têm sido estudadas foram obtidas através de colheitas em pacientes, não é possível garantir que determinadas características estejam, de facto, relacionadas com a resistência ao fármaco, devido à falta de uma estirpe suscetível isogénica para comparação. Tendo em conta esta limitação, no âmbito deste projeto foi desenvolvida uma estirpe de *S. mansoni* resistente ao Praziquantel, que é isogénica à linha parental suscetível que lhe deu origem, exceto no que respeita aos determinantes associados à resistência.

As bombas de efluxo são muitas vezes apontadas como estando envolvidas em falhas de tratamento com outros fármacos. Neste trabalho foi testada a hipótese do envolvimento de bombas de efluxo na resistência ao Praziquantel em *S. mansoni*, através de ensaios de acumulação de Brometo de Etídio na presença de um inibidor de bombas de efluxo (Verapamil), assim como ensaios de suscetibilidade com várias concentrações de fármaco na presença e ausência deste inibidor, e, ainda a realização de qRT-PCR para determinação dos níveis de expressão do gene *SmMDR2*. Comparando os resultados obtidos para cada uma das estirpes, verificou-se que os machos da estirpe resistente apresentam uma maior atividade de bombas de efluxo, uma menor suscetibilidade ao fármaco e uma maior expressão do gene estudado.

Nesta tese também foram estudadas eventuais diferenças morfológicas entre ambas as estirpes. Com recurso a microscopia eletrónica de varrimento, foram observados machos e fêmeas das estirpes suscetível e resistente, na presença e ausência de

Praziquantel. Foi possível constatar a existência de danos significativos no tegumento da estirpe suscetível exposta ao fármaco, o que não aconteceu na estirpe resistente. Além disso, os ovos de ambas as estirpes foram observados por microscopia ótica e constatou-se que os ovos da estirpe resistente são significativamente mais pequenos. O recurso a espectrometria de massa permitiu ainda o estudo do proteoma de machos e fêmeas de ambas as estirpes, sob a ação do fármaco ou não. Este ensaio permitiu caracterizar o proteoma de cada uma das estirpes e identificar 60 proteínas diferentes, algumas das quais expressas de forma diferente entre os grupos.

Assim, tirando partido do desenvolvimento de um novo modelo para o estudo da resistência ao Praziquantel em *S. mansoni* foi possível determinar algumas diferenças existentes entre ambas as estirpes, que possivelmente estarão associadas à resistência ao Praziquantel em *S. mansoni*. Nomeadamente, este trabalho sugere fortemente o envolvimento de bombas de efluxo na resistência ao Praziquantel em *S. mansoni*, sendo necessários mais estudos para avaliar a possibilidade da aplicação de terapias combinadas com inibidores de bombas de efluxo para aumentar a suscetibilidade dos vermes ao tratamento com Praziquantel. As alterações morfológicas observadas são um achado muito importante, pois poderão ocorrer *in vivo* e ter reflexos na biologia do parasita resistente, com impacte na transmissão e patologia da doença, nomeadamente num cenário de neuroschistosomose. Além disso, a elucidação do proteoma de ambas as estirpes será fundamental para trabalhos futuros, representando um grande progresso para o estudo da resistência ao Praziquantel em *S. mansoni*.

Abbreviations list

2-DE	Two-dimensional electrophoresis
ABC	ATP-binding cassette
ABCB1	ATP-binding cassette sub-family B member 1
ABCCs	ATP-binding cassette sub-family C
ACN	Acetonitrile
BH	Belo Horizonte
$CaCl_2$	Calcium Chloride
DMSO	Dimethyl Sulfoxide
dpi	Days post-infection
DTT	Dithiothreitol
EPs	Efflux pumps
EPZQ	Exposed to Praziquantel
EtBr	Ethidium Bromide
FA	Formic Acid
GO	Gene Ontology
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IEF	Isoelectric focusing
IHMT/UNL	Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa
IMPDH	Inosine-5′-monophosphate dehydrogenase
IPG	Immobilized pH gradient
LB	Lower bound
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LD	Lethal dose
LD50	Lethal dose, 50%
LD90	Lethal dose, 90%
LD99	Lethal dose, 99%
MDR	Multidrug resistance
MRPs	Multidrug resistance-associated proteins
MS	Mass spectrometry
MW	Mann-Whitney

$NaHCO_3$	Sodium Bicarbonate
NEPZQ	Not exposed to Praziquantel
Pgp	P-glycoprotein
PKC	Protein kinase C
PZQ	Praziquantel
qRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
RF	Resistant female
RM	Resistant male
RS	Resistant strain
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SF	Susceptible female
SM	Susceptible male
SS	Susceptible strain
TFA	Trifluoracetic Acid
TM	Two transmembrane
UB	Upper bound
Verap	Verapamil

CHAPTER I - INTRODUCTION

I. General introduction

1. The parasite *Schistosoma*

The genus *Schistosoma* belongs to the Phylum Platyhelminthes, Class Trematoda, Subclass Digenea, Order Schistosomatida and Family Schistosomatidae [1]. This genus comprehends six different species, namely, *Schistosoma haematobium, Schistosoma japonicum, Schistosoma mansoni, Schistosoma mekongi, Schistosoma guineensis* and *Schistosoma intercalatum,* all of them causing schistosomiasis. There are two major forms of schistosomiasis – intestinal and urogenital (Table I-1) [2-5].

Table I-1. Parasite species, forms of schistosomiasis caused for these and respective geographical distribution.

	Species	Geographical distribution
Intestinal schistosomiasis	*Schistosoma mansoni*	Africa, Middle East, Caribbean, Brazil, Venezuela and Suriname
	Schistosoma japonicum	China, Indonesia, Philippines
	Schistosoma mekongi	Several districts of Cambodia and Lao People's Democratic Republic
	Schistosoma guineensis *Schistosoma intercalatum*	Rain forest areas of central Africa
Urogenital schistosomiasis	*Schistosoma haematobium*	Africa, Middle East, Corsica (France)

Based on updated and corrected data from: (http://www.who.int/mediacentre/factsheets/fs115/en/ - last accession on February 5th 2016).

Schistosoma spp. comprehends several life stages, namely eggs, miracidium, sporocyst, cercariae, schistosomula and adult worms. The adult schistosomes present a cylindrical body with a complex tegument, as well as two terminal suckers (oral and ventral) (Figure I-1). They are white or grayish worms with about 7 to 20 mm [2, 4, 5], and can live in their mammalian host 3 to 10 years but, in some cases, can survive as long as 40 years [4]. Unlike other trematodes, schistosomes have sexual dimorphism (Figure I-1). Adult male and female worms live much of the time in copula within the perivesical (*S. haematobium*) or mesenteric (all the other species) venous plexus, with the female fixed into the gynaecophoric canal of the male (Figure I-1), where she produces eggs and he fertilizes them. The egg production is dependent on fatty acid oxidation, being those acids obtained from the host [6]. Besides fatty acids, the glucose necessary to get energy from glucose metabolism is also derived from the host by erythrocytes digestion [7]. Schistosomes have a blind digestive tract, thus they cannot

excrete waste products, regurgitating them into the bloodstream. Most of the expelled products are useful for blood-based and urine-based diagnostic assays [4].

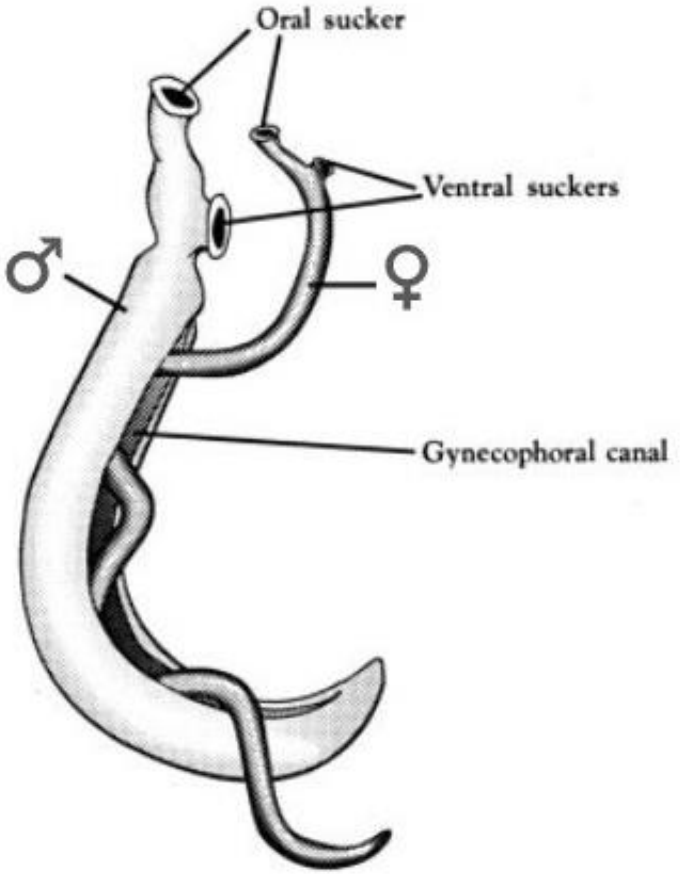

Figure I-1. Paired adult worms of schistosome, showing a female into the gynaecophoric canal of the male, and the oral and ventral suckers of the adult worms, from [8].

The life cycle of *Schistosoma* spp. is shown in Figure I-2. As said above, the adult male and female worms live within the veins of their mammalian host, where they mate and produce fertilized eggs. The females produce hundreds (African species - *S. mansoni, S. haematobium, S. intercalatum* and *S. guineensis*) to thousands (oriental species - *S. japonicum* and *S. mekongi*) of eggs per day. The *Schistosoma* spp. eggs enclose a ciliated larva called miracidium that produces proteolytic enzymes, essential for the eggs migration to the lumen of the bladder (*S. haematobium*) or the intestine (all the other species). The eggs are excreted in the urine or feces of the host, and they can stay viable for up to 7 days [2, 4, 5]. Sometimes inflammation can occur in certain tissues of the host, due to the deposition of eggs in these locations, rather than being excreted. On the other hand, the eggs that reach freshwater will hatch, releasing miracidium that then infect a suitable snail host (for example *Biomphalaria glabrata* for

S. mansoni). In its intermediate host, the parasite undergoes asexual replication through mother and daughter sporocyst stages, eventually shedding tens of thousands of cercariae (the infectious form for the mammalian host) into the water. This part of the life cycle in snails requires 4 to 6 weeks before the release of cercariae. Once cercariae penetrate the skin of the mammalian host, the maturing larvae called schistosomula need about 5 to 7 weeks before becoming adults and producing eggs. These periods in both the intermediate and in definitive hosts, during which the infection is ongoing but release of cercariae from snails or eggs from mammals cannot be detected, are denominated pre-patent periods. Contrarily to what happens to eggs, that die 1 to 2 weeks after female worm release them, cercariae can only stay infective in freshwater during 1 to 3 days, even though they deplete the most of their energy reserves in a few hours [2, 4 ,5].

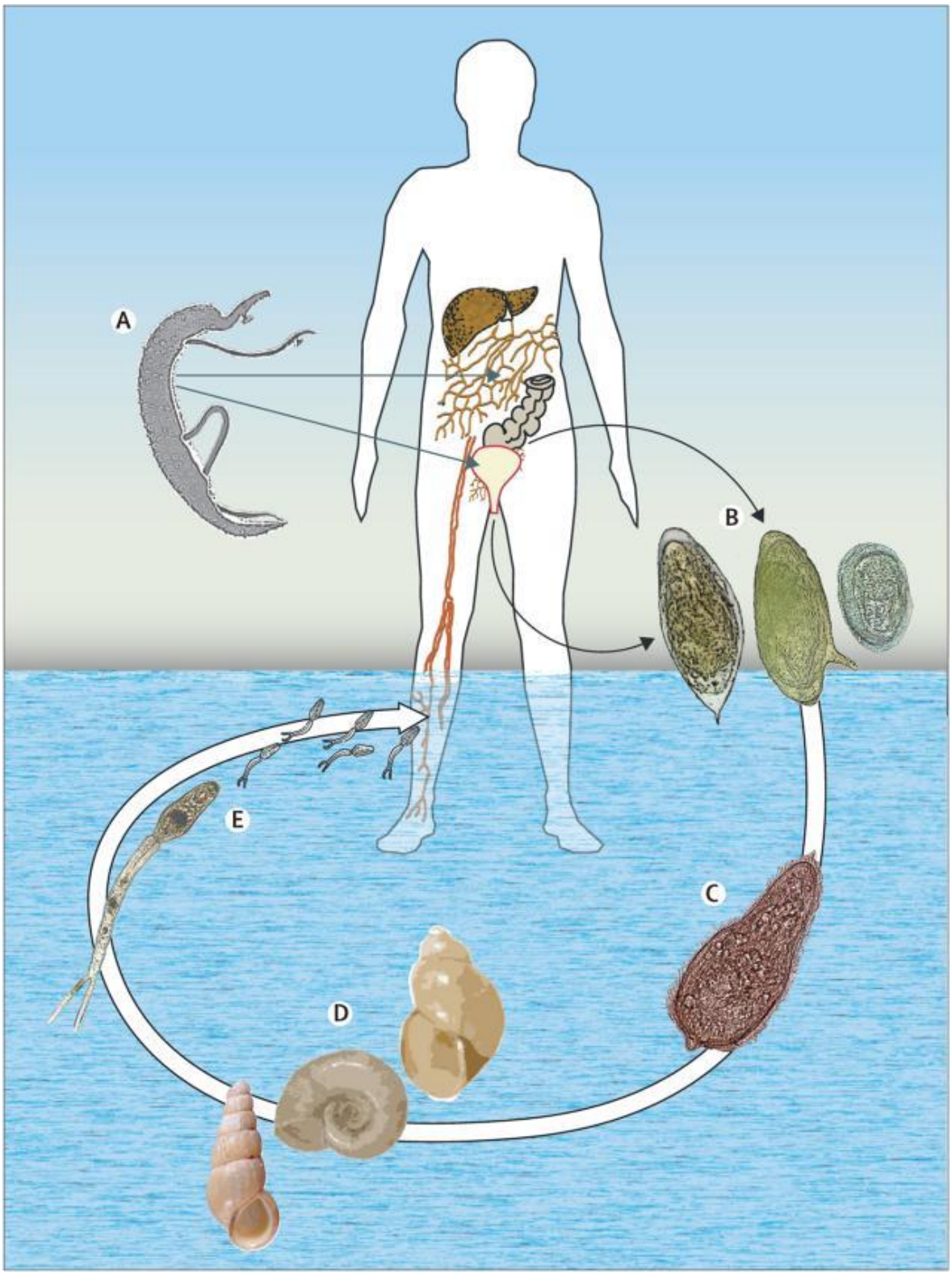

Figure I-2. Transmission cycle of the three main species of *Schistosoma: S. haematobium, S. mansoni* and *S. japonicum*. A: Paired adult worms (male holding female). B: Eggs (left to right, *S. haematobium*, *S. mansoni*, *S. japonicum*). C: Ciliated miracidium. D: Intermediate host snails (left to right, *Oncomelania*, *Biomphalaria*, *Bulinus*). E: Cercariae. From [4], image adapted from [2].

Transmission invariably occurs when people and other mammals suffering from schistosomiasis contaminate freshwater with their excreta containing parasite eggs, which hatch in freshwater. People become infected when infectious cercariae penetrate the skin during contact with freshwater, usually by swimming or washing [9].

Schistosoma japonicum and *S. mekongi* schistosomiasis are zoonosis that, besides humans, also infect a broad range of mammalian hosts, as dogs, pigs, or cattle, greatly complicating the control and elimination efforts. Rodents and non-human primates can also be infected by *S. mansoni* and *S. haematobium,* but they are not the predominant reservoir of this specie. To successfully control and eliminate human schistosomiasis, it is very important to have a good knowledge about the life cycle and the transition of the parasite between intermediate and definitive hosts [2, 4, 5].

2. History of schistosomiasis

The earliest description of schistosomiasis can be found in the ancient Assyro-Babylonian literature, namely in the *Papyrus Ebers* of Egypt [10], where it is mentioned a worm disease that seems to be related with urinary bleeding (possibly a *S. haematobium* infection). However, any systematic effort was performed to understand the basic life cycle and pathogenesis of this disease until the XIX century [8].

The first description of *Schistosoma* spp. is attributed to Theodor Bilharz, who during the autopsy of a young man found worms in the portal vein [11]. This German pathologist also described the presence of eggs with a spine, which indicates that he could be in presence of *S. mansoni* or *S. haematobium*, but makes impossible the distinction of those two species, since this is a common characteristic (Figure I-2). Those findings were communicated to his professor von Freholdtz in 1851, and two years later presented in a meeting at Breslau. Bilharz also described the characteristic pathologic changes and clinical features of schistosomiasis, and named those worms *Distomium haematobium* [12, 13]. In 1858, Wienland and Cobbold disagreed with this nomination because of the observation that only one of the two suckers of these worms led into the oral cavity, and suggested the name *Schistosoma* [14, 15], where “Schisto” describes the slit in the male and “soma” the body of the enveloped female worm. Therefore, since 1864 the official nomenclature to this disease is "schistosomiasis", but in Europe and the Middle East the term “bilharzia” has been commonly used [8].

3. Schistosomiasis

Schistosomiasis or bilharzia is one of the major parasitic diseases of the tropics [2, 4, 5]. As said in section 1 of this Chapter, this disease is caused by several species of *Schistosoma* - trematode parasites, also known as blood-dwelling fluke worms [16]. There are three main species infecting human beings, namely, *S. haematobium*, *S. mansoni*, and *S. japonicum*. *Schistosoma haematobium* and *S. mansoni* both occur in Africa and the Middle East, whereas only *S. mansoni* is present in the Americas. *Schistosoma japonicum* is localized in Asia, primarily the Philippines and China. The other three species, *S. mekongi, S. guineensis,* and *S. intercalatum* are more locally distributed, the first one in the Mekong River basin and the other two in West and Central Africa (Figure I-3), and they also cause human disease. Each of those species has specificity to its suitable snail host, which makes their distribution be defined by their host snails' habitat range [4].

With over 249 million individuals infected with schistosomiasis worldwide, more than 97% are in sub-Saharan Africa (Figure I-4). About 120 million of all people infected have symptoms and 20 million have severe consequences. This parasitosis causes about 280 000 deaths annually and around 732 million people are at risk of infection in 78 countries [17-19].

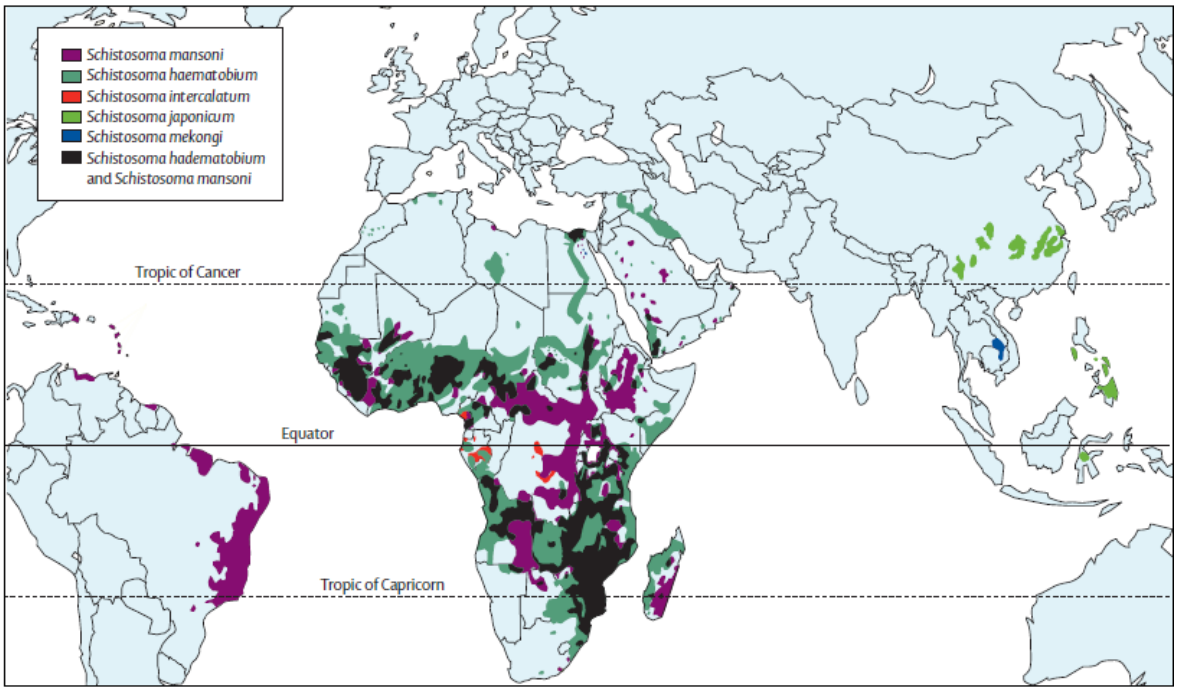

Figure I-3. Global distribution of human schistosomiasis transmission. From [3], modified from [2, 17].

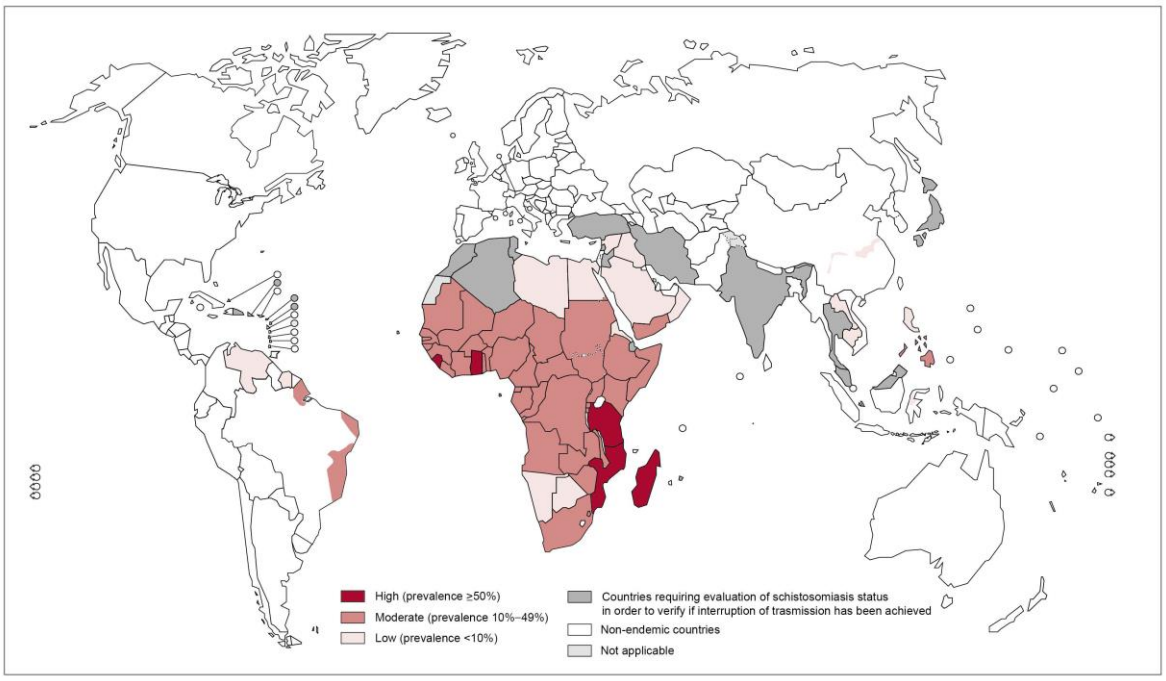

Figure I-4. Worldwide distribution of schistosomiasis, taking into account the prevalence rate, from [20].

In Europe, schistosomiasis occurs due to the arrival of infected immigrants, and schistosomiasis in returning travelers is one of the most common imported tropical infections with potentially serious acute and long-term complications [5, 21]. In 11 years (1997-2008), more than 400 cases of travel-associated schistosomiasis were reported by the Geosentinel Surveillance Network in 27 sites from 12 Western countries [22]. Most cases of imported schistosomiasis in Europe are described in travelers returning from sub-Saharan Africa [22], and only rare cases are still imported from Asia and Latin America, possibly due to the reduced incidence in those continents as a result of successful schistosomiasis control programs, most notably in China [5, 21].

3.1. Clinical presentation

As described in [5], schistosomiasis presentation can be divided into several syndromes:

1) Asymptomatic/non-specific are the cases where some patients can only report low grade fever, dermatitis or pruritic rash (commonly called “swimmers itch”) at the site of infection after cercariae penetrating the skin. It is very important identifying these patients at an initial stage of infection, because an early diagnosis can prevent long-

term complications [23]; 2) Acute schistosomiasis is an immune complex-mediated response to the immature forms of the parasite migrating through the body, and it presents fever, malaise, urticarial, wheeze, hepatosplenomegaly and eosinophilia, usually appearing 4 to 8 weeks after parasite exposure; 3) Hematuria is very common in patients infected by *S. haematobium.* As this species is responsible for urogenital schistosomiasis, gynecological presentations with vulva schistosomiasis [24] or hematospermia or lumpy semen may also occur. Those situations have a slightly increased risk of HIV transmission; 4) Diarrhea can occur in patients with schistosomiasis, but this disease rarely cause chronic diarrhea. Patients with a combination of diarrhea and eosinophilia should immediately be questioned about tropical travel; 5) Chronic infection is a very serious situation where patients can present permanent scarring of the bladder, liver and urogenital system, due to eggs retained in those tissues. In the most severe situations with heavy chronic infections the risk of bladder carcinoma (*S. haematobium*), or liver scarring and/or portal hypertension with ascites (*S. mansoni* or *S. japonicum*) is increased; 6) Neuroschistosomiasis occurs with the egg deposition in the central nervous system, namely in brain or spinal cord, and this is a very rare but important syndrome [5].

Neuroschistosomiasis should be seen as a severe condition where the prognosis is mainly dependent on an early treatment, and consequently on an early diagnostic [3, 25-29]. This syndrome is probably under-recognized, because besides rare by comparison with intestinal or urogenital cases, it is not uncommon. *Schistosoma mansoni*, *S. haematobium* and *S. japonicum* are responsible for almost all reported cases of neuroschistosomiasis [3, 29, 30], with *S. mansoni* and *S. haematobium* usually associated to the spinal cord, and *S. japonicum* to encephalic disease [3, 27-29]. In most symptomatic patients, the occurrence of nerve damage depends on the presence of parasite eggs in the nervous tissue and on the host immune response [3, 29].

3.2. Diagnosis

Except for acute schistosomiasis, the finding by microscopy of *S. haematobium* eggs on filtered urine and eggs from all other species on stool, is the gold standard for diagnosis of schistosomiasis. However, in light infections and in travelers from endemic countries, this method has low sensitivity. Therefore, serology for schistosome

antigens is the standard screening method in these situations, which has a sensitivity of over 90% [5].

An important step on schistosomiasis diagnosis is the knowledge of patient historical. Suspected individuals or travelers returning from endemic areas should be questioned about freshwater exposure, positive serological tests, and history of cercarial dermatitis. This might be indication of infection, but the absence of these indicators does not avoid the need of further investigations. So it is very important take in account in clinical practice that, neither a negative serological test nor the absence of eggs in urine or stool is sufficient to rule out schistosomiasis [3].

3.3. Treatment

The standard treatment of schistosomiasis is with Praziquantel (PZQ), giving a dose of 40 or 60 mg/kg bodyweight orally. In heavy infection or for acute cases, the treatment may need to be repeated, as PZQ does not effectively treat immature schistosomes. In addition corticosteroids can also be given during acute phase to alleviate symptoms. Follow up of schistosomiasis patients is only advantageous if eggs or eosinophilia were found, in order to make sure the resolution within approximately 3 months. Antibody serology for schistosomiasis may remain positive for years following successful treatment, and therefore it is not useful for follow up. Most cases of schistosomiasis, once treated, will cause no long-term sequel, although there should be taken into account the investigation of patients with a history of schistosomiasis and hematuria with cystoscopy, due to the risk of bladder carcinoma in cases of prolonged *S. haematobium* infection [5]. Besides antischistosomal drugs, corticosteroids, and surgery are used to treat neuroschistosomiasis cases [26-28].

3.4. Disease control

Vaccines and prophylactic treatment are not available to prevent infections with *Schistosoma* spp.. Thus, to prevent schistosomiasis transmission, it is essential apply effective treatment to infected people, prevent sewage contamination of freshwater, eliminate the intermediate host snails, and avoid human contact with water containing infected snails [4]. Besides, environmental changes can either increase or decrease transmission [31, 32]. Alterations not only in snail habitat, but also their predators, are determinant for transmission [33].

The advances in schistosomiasis control with the massive use of PZQ in endemic areas resulted in substantial decreases in morbidity and mortality of this disease, but were not sufficient to stop its geographical expansion, and schistosomiasis continues spreading to new regions [34, 35]. Environmental changes resulting from the industrialization of developing countries and migration of populations are thought to be behind the continued spread of the infection [31, 34, 36]. Moreover, the number of travel-related schistosomiasis reports has been increasing [37].

The outcomes of the schistosomiasis control programs are considered disappointing [38]. In fact, decades of mass PZQ administration have had negligible effects on the global prevalence of schistosomiasis. In certain regions, the disease has expanded to new foci [39]. Less than 5% of the schistosomiasis-affected population is treated with PZQ. With such this low coverage, it will be difficult for any program to make significant contributions to schistosomiasis control. Since PZQ does not prevent re-infection, drug administration needs to be constant in order to maintain disease control [40].

PZQ has been used so extensively and so exclusively [41], that it is important to have in mind that the trap of an excessive medicalization should be avoided. Schistosomiasis is a poverty disease, so its full control could be achieved, in principle, just by removing the socio-economic causes that lay at its basis. The often-recommended integrated approach to control schistosomiasis should comprise, among other measures, sanitation, water supply, ecological interventions and health education. In the transmission of schistosomiasis, Man almost appears as a vector, because the transmission would be interrupted, at least in regions without non-human hosts, if people avoided urinating or defecating in or near water bodies [41].

In this context of poverty, the impossibility of alternative approaches as vaccination, and the difficulties in attack the snails populations acting as intermediate hosts, make chemotherapy the main approach for schistosomiasis control. In the last decades, several PZQ mass administration programs have been applied to millions of people every year, since this is the only available drug to treat this disease. This massive and exclusive use of this drug for so much time brought concerns about the rise of PZQ-resistant schistosomes, and those concerns are on theory legitimated by what happened in the past with other anti-infective agents [41].

4. Praziquantel and resistance

PZQ was developed in the 1970s and shortly referenced by the World Health Organization as the treatment of choice for schistosomiasis, and began to be marketed for human use as Biltricide [42]. PZQ is the generic name for 2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinoline-4-one, which is a white crystalline powder with a bitter taste, normally stable under normal storage conditions, practically insoluble in water but soluble in some organic solvents. The commercial preparations of the drug are usually racemic mixtures composed of equal portions of the “laevo” and “dextro” isomers, of which only the “laevo” form has schistosomicidal activity [42-44].

PZQ is first metabolized during its passage through the liver and it disappears relatively rapid from the circulation with a half-life of 1-1.5 h, and the elimination from the body is rapid and mainly via urine, the remainder via feces, and is largely complete after 24 h. Animal tests have demonstrated that PZQ has very low toxicity and no genotoxic risks were detected in assays for mutagenicity or carcinogenicity [42].

The mechanism of action is not exactly known at present, but experimental evidence indicates that PZQ kills adult worm schistosomes by increasing the Calcium permeability of the tegument membrane, thereby, disrupting Calcium homeostasis and causing paralysis of the worms [45]. PZQ is considered an excellent drug and has several advantages, mainly regarding safety, efficacy and cost, but the lack of efficacy against *Schistosoma* spp. immature forms is its potential significant limitation [41, 42].

As said in section 3.4 of this Chapter, the massive programs performed raise concerns about PZQ-resistance in *Schistosoma* spp.. In fact there has already been some observed decrease of PZQ susceptibility in some strains [46]. Therefore, the research of new antischistosomal drugs is imperative and urgent. Although a large number of compounds have been tested for its potential activity against schistosomes (some of them seeming promising), until now there is not an alternative or adjunct compound to PZQ. The uncertain about the mechanisms of action of this drug is not favorable to the development of alternative drugs to treat schistosomiasis, and therefore it is also important to better characterize this drug and its mechanisms of action [41].

Resistance to PZQ is defined as the genetically transmitted loss of susceptibility in worm populations that were previously susceptible to PZQ. In this process,

chemotherapy selectively removes susceptible worm individuals from the genetically heterogeneous populations leading to an increase in individuals carrying genes conferring drug resistance that are passed to the offspring generation. Over several generations, resistance genes accumulate so that a large number of worms within a population survive following treatments. It is believed that the transmission of genetic material only occurs through parental lineages, as there is, as yet, no evidence for transposition of genetic components in this process [47].

Research into the biochemical, genetic and proteomic mechanisms of drug resistance have been shown to be of great relevance for drug target identification and epidemiological studies [48]. The repetitive exposure to sub-lethal doses of PZQ in mice have generated resistant strains of *S. mansoni* in 2 generations [49], and field and laboratory isolates with reduced susceptibility or possible resistance to PZQ have already been identified [42-43, 50-56], thus demonstrating that resistance is more than a hypothetical possibility, though no gene(s) has been unequivocal associated with this phenotype.

The less efficacy of PZQ treatment of schistosomiasis appeared about 10-15 years after the application of this treatment in a massive scale in Egypt and after the introduction of this disease in Senegal [46, 47, 57]. The longtime of exposure before appearance of resistance, suggests that PZQ resistance might be a multiple gene phenomenon [58]. In the both cases, the less susceptibility of worms obtained from uncured patients was also observed in mouse model [59], which may indicate that the PZQ failure is related to worms characteristics. But other factors as host factors, heavy worm burdens, and pre-patent infections may also be involved in the treatment failure [47, 59]. The difficulties verified in treat travelers with schistosomiasis further emphasize the need to remain vigilant [59]. So far, to our knowledge, no full and unequivocal association has been made between PZQ-resistance and a gene or a genetic trait.

5. Efflux pumps

One of the more common mechanisms for development of drug resistance is through increased drug efflux, often mediated by multidrug transporters. Multidrug transporters are involved in multidrug resistance (MDR), which is a phenomenon where besides the resistance to a drug, there is an unexpected cross-resistance to several other compounds, even structurally unrelated [60]. Multidrug transporters are responsible for remove xenobiotics and toxic compounds, including drugs, from cells and tissues, and are considered to have low substrate specificity [61]. Furthermore, the efflux mechanisms are currently known to be the major components of resistance to many drugs, particularly to antibiotics. [62, 63]. There are certain efflux pumps (EPs) that extrude specific antibiotics, but there are those called multidrug EPs, that extrude a diversity of compounds with diverse functionality and structure [64]. An excellent approach used nowadays to deal with MDR is undoubtedly the use of inhibitors of EPs, thus improving the clinical performance of many drugs [65]. The Figure I-5 shows a list of some substrates and inhibitors commonly identified.

Chemotherapy failure in the treatment of bacteria and cancer has been associated to the activity of ATP-binding cassette (ABC) transport proteins. The ABC-transport proteins belong to a family of membrane proteins that play important roles, such as transport of diverse compounds (peptides, hormones, cholesterol and iron), in both eukaryotes and prokaryotes [66, 67]. However, several members of this family of drug transporters, as P-glycoproteins (Pgp) (Figure I-5) and the multidrug resistance-associated proteins (MRPs) in particular, might be involved in drug resistance in parasites [68-70]. PZQ may interact with Pgp or MRPs in a number of ways, as either a substrate or as an inhibitor of transport mediated by the ABC-transport proteins [71].

Most of the information regarding ABC-transporters and their involvement in MDR is based on studies of Pgp, an organic cation pump that is the product of *ABCB1* gene. It is a full transporter comprised of 12 transmembrane segments divided into two transmembrane (TM) domains, each linked with an ATP-binding domain (Figure I-5) [72].

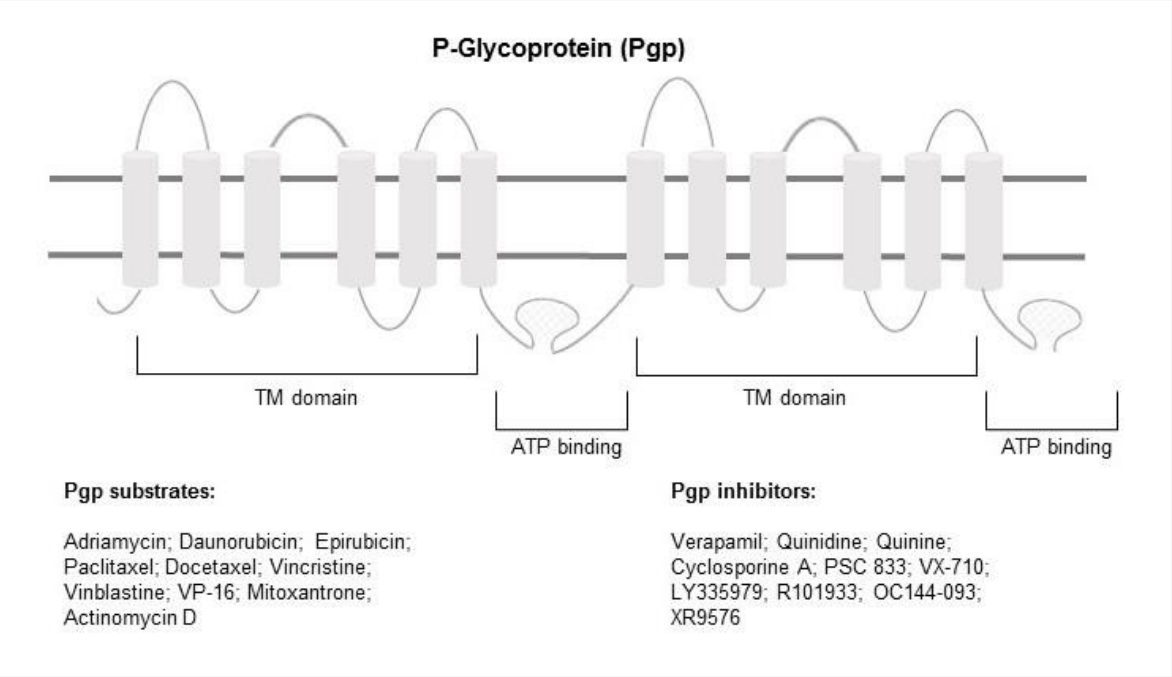

Figure I-5. P-Glycoprotein (Pgp) structure and list of substrates and inhibitors, adapted from [72].

The activity of EPs of prokaryotes and eukaryotes can be inhibited with phenothiazines [73]. The mechanism by which phenothiazines affect EPs activity is by inhibiting ATPases [74]. Phenothiazines are known to reverse resistance of a number of human parasites [74]. Since phenothiazines inhibit EPs, resistance of parasites to antiparasitic drugs may, as it is the case of most micro-organisms, be due to an over-expression of EPs. Indeed, there are supporting evidences that drug resistance of helminths involves EPs [73-75].

Two homologues ABC-transport protein have been identified in *S. mansoni*: *SmMDR1* and *SmMDR2*. *SmMDR1* has no known homologues, whereas *SmMDR2* is homologous to mammalian Pgp (MDR1) [76]. There are indications that multidrug transporters may be involved in modulating levels of PZQ susceptibility in schistosomes [60]. PZQ is both an inhibitor and a substrate of recombinant *SmMDR2* [77], and chronic exposure of worms to sub-lethal concentrations of PZQ results in up-regulation of *SmMDR2* and *SmMRP1*, and changes the distribution of anti-Pgp immunoreactivity in the worm [78, 79]. Importantly, higher levels of schistosome *SmMDR2* and *SmMRP1* are associated with reduced PZQ susceptibility [78, 79]. Indeed, an Egyptian isolate with reduced PZQ susceptibility, expresses dramatically higher levels of *SmMDR2* [78].

6. Schistosome proteome

Research of schistosome proteome might be extremely important for the understanding of immune mechanism, investigation of new diagnostic and vaccine candidates, as well as the development of new drugs [80].

The enlargement of schistosome databases alongside the upgrading of technology in this area, helped to improve the schistosome proteome studies. However, there is still limitations in finding much schistosome proteins that have high homology with proteins from other organisms, because there is no organisms so phylogenetically close to schistosome that has good protein sequences available in the public databases. So the sequencing of schistosome transcriptome came greatly improve the level of identification in proteomics studies, as well as the reliability of protein identification associated with the orthologous proteins from public databases, because it was possible to generate large databases of *Schistosoma* coding sequences [81, 82].

The most proteomics studies in schistosome are done in order to compare the amount of proteins at different stages of the life cycle of the parasite, which may enable to identify proteins with specific functions in each stage of the life cycle. [83]. In a study done by Curwen and colleagues [84], they compared soluble extracts from three different stages of *S. mansoni* life cycle, and the most abundant proteins identified for them were characterized for having high immunogenicity and thus considered as good vaccine candidates. Analysis of protein extracts from various life stages of schistosome reveled proteins with specific stage functions, and these proteins were considered good candidates to be promising therapeutic targets [85].

Other proteomics studies in schistosome have discovered gender-specific proteins involved specifically in sexual maturation, in reproduction, and in hormone receptors. These proteins are considered of great importance in the disease control as they may be used to block egg laying, which may reduce pathology induced by eggs and also reduce the disease transmission [85, 86].

Proteomics analysis is considered a powerful tool to screen samples derived from pathogens and identify proteins that are possibly involved in pathogenesis [87]. In the case of schistosome studies, it is used to identify proteins from complex samples (tegument and secretion proteins) or to study differential expression of proteins [84, 86, 88-91]. This approach has also been used to analyze protein expression in adult

schistosomes from susceptible, less susceptible or resistant hosts, helping to identify essential molecules involved in the survival and development of the parasite, and perhaps new vaccine candidates or even drug targets useful for the schistosomiasis control [87].

7. Schistosome tegument

In contrast to nematodes, schistosomes are covered by a living syncytium, called tegument (Figure I-6), instead of cuticle [92]. The schistosome tegument is delimited by an invaginated plasma membrane at its basal surface, while its apical surface has an unusual hepatalaminate appearance, which is considered the normal plasma membrane, covered by a membrane-like secretion, called membranocalyx (Figure I-6) [92].

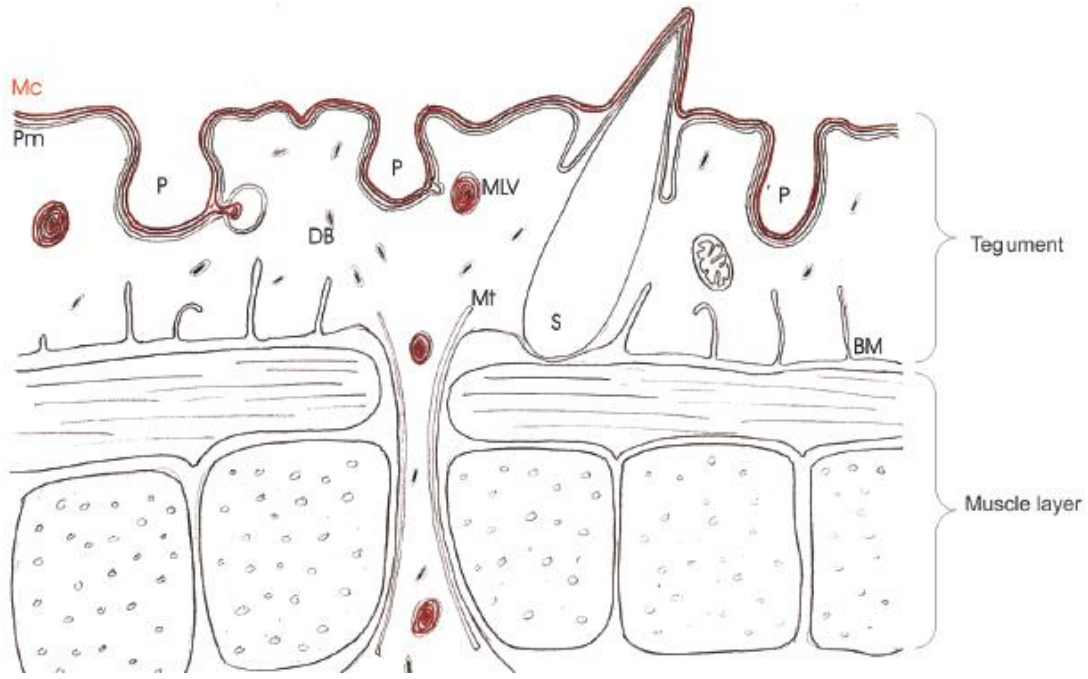

Figure I-6. Illustrative representation of the schistosome tegument. Mc: Membranocalyx; Pm: Plasma membrane; P: Pits; DB: Discoid body; MLV: Multilaminate vesicle; Mt: Microtubule; S: Spine; BM: Basal membrane, adapted from [92].

One of the most important organ of schistosome parasites is the tegument, which has been immensely studied, once it plays a crucial role on the protection of the parasite against the action of immune system of the host, helping the adult worms to survive in the host, since it is renovated in just a few hours [93-96]. Other important roles of this

organ are related to its ability to absorb nutrients and molecules and synthesize some proteins [97-99].

The advent of microscopy techniques used to analyze the worms' structure, contributed to the knowledge of the importance of schistosome tegument, and also allowed distinguishing between male and female worms of *Schistosoma* spp., like the fact that males have more and larger thorns than females. Therefore the emergence of transmission electron microscopy and scanning electron microscopy (SEM) has revolutionizing the knowledge of the adult worms of schistosomes and the description of its sexual dimorphism [96].

As said before, schistosome adult parasites can live about 3 to 10 years in their definitive mammalian host, mostly due to their ability to escape the immune-mediated responses [100]. Some features of these parasites as the peculiar structure of their tegument and their capacity to penetrate the immune system, facilitate them to stay alive within the blood vessels environment for so long [101].

In addition to all the important features and functions already mentioned, the tegument helps the parasite interacting to the host, and also participates in the process of excretion, osmoregulation and signal transduction [102, 103]. This great organ plays another major role in helping the parasite at different stages of the life cycle, as assisting them to transform from the free-living stage (miracidium and cercariae) into the parasitic stage (schistosomula and adult worms), which allow the parasite to migrate through various environments in the mammalian host. Schistosome parasites can do these by switching the composition of their tegument in a short time. Indeed, this characteristic allows the parasite switch from an immune-sensitive to an immune-refractory state [102, 104].

8. Background and research objectives

In the last years we have developed in the laboratory at Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT/UNL) a *S. mansoni* PZQ-resistant strain (IHMT/UNL) in mice [56] derived from the PZQ-susceptible BH (Belo Horizonte, Minas Gerais, Brazil) *S. mansoni* parasite strain, placing us in an excellent position to perform direct comparisons between the PZQ-resistant and the parental PZQ-susceptible strains. The knowledge of possible mechanisms involved in resistance to PZQ will allow a better assessment of the epidemiology of drug-resistance to PZQ in the field also enabling a more effective decision of, for example, a potential drug to use in combination with PZQ. Having this in mind, the main objectives of this thesis are:

1- Evaluate the role of EPs in *S. mansoni* PZQ-resistance phenotype, by comparing the EPs activity in the PZQ-susceptible and the PZQ-resistant parasite strains, upon exposure to a compound known to inhibit eukaryotic EPs – Verapamil (*Chapter II*);

2- Develop a new methodology allowing the study, on a real time basis, of the transport of the universal efflux substrate Ethidium Bromide (EtBr) and to correlate the efflux inhibitory effects with the resistant variant (*Chapter II*);

3- Evaluate morphological alterations in the *S. mansoni* PZQ-resistance phenotype by comparing the PZQ-resistant strain obtained under PZQ drug pressure with the PZQ-susceptible strain (*Chapter III*);

4- Analyze the proteome of *S. mansoni* PZQ-resistant adult worms and compare it with its parental fully PZQ-susceptible strain, using a high throughput Liquid chromatography-tandem mass spectrometry (LC-MS/MS) identification (*Chapter IV*).

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CHAPTER II – RESEARCH WORK 1

II. The role of efflux pumps in *S. mansoni* Praziquantel resistant phenotype

Adapted from: Pinto-Almeida, A., Mendes, T., Armada, A., Belo, S., Carrilho, E., Viveiros, M., and Afonso, A. 2015. The role of efflux pumps in *Schistosoma mansoni* Praziquantel resistant phenotype. *PLoS ONE*. 10(10):e0140147. doi:10.1371/journal.pone.0140147.

1. Abstract

Schistosomiasis is a neglected disease caused by a trematode of the genus *Schistosoma* that is second only to malaria in public health significance in Africa. PZQ is the drug of choice to treat this disease due to its high cure rates and no significant side effects. However, in the last years increasingly cases of loss of sensitivity to PZQ have been reported, which has caused growing concerns regarding the emergence of resistance to this drug. Here we describe the selection of a parasitic strain that has a stable resistance phenotype to PZQ. It has been reported that drug resistance in helminths might involve EPs such as members of ABC-transport proteins, including Pgp and MRPs families. Here we evaluate the role of EPs in *S. mansoni* resistant to PZQ, by comparing the EPs activity in susceptible and resistant strains. The evaluation of the efflux activity was performed by an EtBr accumulation assay in presence and absence of Verapamil. The role of EPs in resistance to PZQ was further investigated comparing the response of susceptible and resistant parasites in the absence and presence of different doses of Verapamil, in an *ex vivo* assay, and these results were further reinforced through the comparison of the expression levels of *SmMDR2* by qRT-PCR. This work strongly suggests the involvement of Pgp-like transporters *SmMDR2* in PZQ drug resistance in *S. mansoni*. Low doses of Verapamil successfully reverted drug resistance. Our results give an indication that a combined therapy with PZQ and natural or synthetic Pgp modulators can be an effective strategy for the treatment of confirmed cases of resistance to PZQ in *S. mansoni*.

2. Introduction

Schistosomiasis is a neglected tropical disease that affects approximately 249 million people worldwide, 97% of which are located on the African continent. It ranks, with malaria and tuberculosis, as a major source of morbidity despite strenuous control efforts [1, 2]. Furthermore, amongst all the parasitic diseases, schistosomiasis is one of the most common human parasitic diseases whose socioeconomic impact is only surpassed by malaria [2, 3]. Schistosomiasis is caused by blood flukes of the genus *Schistosoma*, which have a complex life cycle comprising a vertebrate host and an invertebrate host. *Schistosoma mansoni* is one of the species that infects humans [4–6] and one of the most common etiological agent for human schistosomiasis, causing more than 83 million human infections in 54 countries [7].

Schistosomiasis treatment relies almost exclusively on the anthelmintic PZQ. However this drug does not prevent reinfection and, with large-scale control programs promoting the extensive use of PZQ for more than 20 years in some African nations, concern regarding the selection of drug resistant parasites has been raised [8–10].

Resistance to PZQ is defined as the genetically transmitted loss of susceptibility in worm populations that were previously susceptible to PZQ. In this process, chemotherapy selectively removes susceptible individuals from the genetically heterogeneous populations leading to an increase of individuals carrying genotypic determinants conferring drug resistance that are passed to the offspring. After several generations, a large number of worms within the population survive following treatment [10].

In vivo artificial selection in mice has previously produced PZQ-resistant lines of *S. mansoni* in only two generations after repeated exposure to sub-lethal doses of the drug [9], demonstrating that resistance is more than an hypothesis. Low cure rates in response to PZQ emerged 10–15 years ago after mass scale use in countries like Egypt and Senegal [11, 12]. Worms from the non-cured patients were repeatedly less susceptible to PZQ when tested in a mouse model [13]. Worm genetic determinants for resistance led to PZQ failure, although host factors, among other factors, were also considered to have contributed to PZQ failure such as heavy worm burdens and pre-patent infections [10, 13]. Difficulties in obtaining cure among travelers with

schistosomiasis [13] further emphasized the need to maintain surveillance in order to avoid parasite spread to places where the intermediate host is present.

Chemotherapy failures in bacteria and cancer treatments have been associated to the activity of ABC-transport proteins [12, 14]. ABC-transport proteins are a large family of membrane proteins that have many multiple cellular functions including the transport of diverse compounds such as peptides, hormones, cholesterol and iron [14, 15]. Several members of this family also transport drugs, such as the P-glycoprotein (Pgp, ABCB1) and the multidrug resistance-associated proteins (MRPs, ABCCs), both reported to be involved in drug resistance by exporting drugs to the outside of parasites either by increased efflux activity or genetic over expression. PZQ is hypothesized to interact with Pgp-like or MRPs either as an efflux substrate or as a competitor of transport mediated by the ABC-transport proteins of other efflux substrates [16]. It has been demonstrated that the activity of EPs of prokaryotes and eukaryotes can be inhibited by Calcium channel blockers, such as Phenothiazines or Verapamil as they inhibit the transporter associated ATPases, such as those that provide the energy for the activity of Pgp-like EPs [16–19].

ABC transporter cDNAs that have been characterized in schistosomes includes *SmMDR2* [20], a *S. mansoni* orthologue of Pgp, and *SmMRP1* [21], a *S. mansoni* orthologue of MRP1. *SmMDR2* is expressed at higher levels in female parasites than in males [20, 22], while male express higher *SmMRP1* levels than females [21]. Notably, adults of *S. mansoni* up regulate the expression of both of these transporters in response to PZQ. Furthermore, higher basal levels of both *SmMDR2* and *SmMRP1* correlate with reduced PZQ susceptibility [21, 22], and PZQ inhibition activity, likely also a substrate of *SmMDR2* [19]. Based on these findings, Kasinathan and colleagues have hypothesized that *Schistosoma* MDR transporters may be modulating the responsiveness of parasites to PZQ [18].

In this study, we have selected from the fully susceptible parasite BH strain, by stepwise drug pressure, a *S. mansoni* variant strain that is stably resistant to PZQ. This resistant parasite variant strain, obtained from infected mice, tolerates up to 1,200 mg PZQ/kg of mouse bodyweight and is isogenic to its parental fully susceptible counterpart, except for the genetic determinants accounting for the PZQ-resistant phenotype. This resistant parasitic strain enabled us to further test the hypothesis that EPs play an important role in the development of the PZQ-resistant phenotype in *S.*

mansoni. Therefore, the aim of this study was to evaluate the role of EPs in *S. mansoni* PZQ-resistant phenotype, by comparing the EPs activity in the PZQ-susceptible and the PZQ-resistant parasite strains, upon exposure to a compound known to inhibit eukaryotic EPs - Verapamil. A new methodology was also developed allowing the study, on a real time basis, of the transport of the universal efflux substrate EtBr and to correlate the efflux inhibitory effects with the resistant variant, which over-expresses Pgp-like EPs demonstrating their important role on PZQ-resistance in *S. mansoni*.

3. Material and Methods

3.1. Reagents

The inhibitor Verapamil, EtBr and Calcium Chloride ($CaCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PZQ was purchased from Merck & Co. (Kenilworth, NJ, USA) and dissolved in 1% of Dimethyl Sulfoxide (DMSO) from Sigma-Aldrich, used for stock solutions, which were subsequently diluted to an appropriate concentration in culture media. All solutions were prepared in distilled, sterile water, on the day of the experiments.

3.2. Animal model

The PZQ-resistant parasitic strain of *S. mansoni* was developed using *Mus musculus* CD1 line males, approximately eight weeks old. CD1 is considered the animal model of choice for *S. mansoni* infection, because it is a good host for this parasite mimicking the *S. mansoni* human infection [23].

The mice used, weighting around 20 g, were obtained from the animal breeding facility (bioterium) of IHMT/UNL. They were kept in appropriate conditions of temperature (±21° C), humidity (45–55%) and light (12 h cycles of light/darkness). The infection occurred by exposing mice tails to about 100 cercariae of *S. mansoni* each, thus mice infection occurred by natural transdermal penetration of the cercariae.

3.3. Parasite isolation

In this study we used a *S. mansoni* BH line, susceptible to PZQ. This parasitic line is routinely kept in our group at IHMT/UNL, using *B. glabrata* as intermediate host.

Our stable PZQ-resistant parasite strain was obtained from the BH line submitted to various steps of PZQ continuous drug pressure, starting with a sub therapeutic PZQ dose and finishing with 1,200 mg/kg of PZQ. Cioli and colleagues [13] demonstrated that in the murine model we can define a line of *S. mansoni* as resistant if it has a LD50 greater than 100 mg/kg, therefore our variant strain resists to 12 times this value.

Infected CD1 mice were checked approximately 60 days post parasite infection by Kato-Katz procedure. If eggs were found in feces, mice were then treated orally with PZQ solution at appropriate dose. If, on day 15, post PZQ treatment, viable eggs (verified by live miracidium inside the eggs and Kato-Katz procedure) continued to be

eliminated, mice were euthanized and eggs present in the liver were used to obtain miracidium to subsequently infect *B. glabrata* snails. Once *B. glabrata* snails start eliminating *S. mansoni* cercariae (30 to 60 days after snail infection), new CD1 mice were re-infected and the previous procedure was repeated, continuing the PZQ-resistant strain selection *in vivo* (Figure II-1). PZQ dose was increased every two passages as shown in Figure II-2.

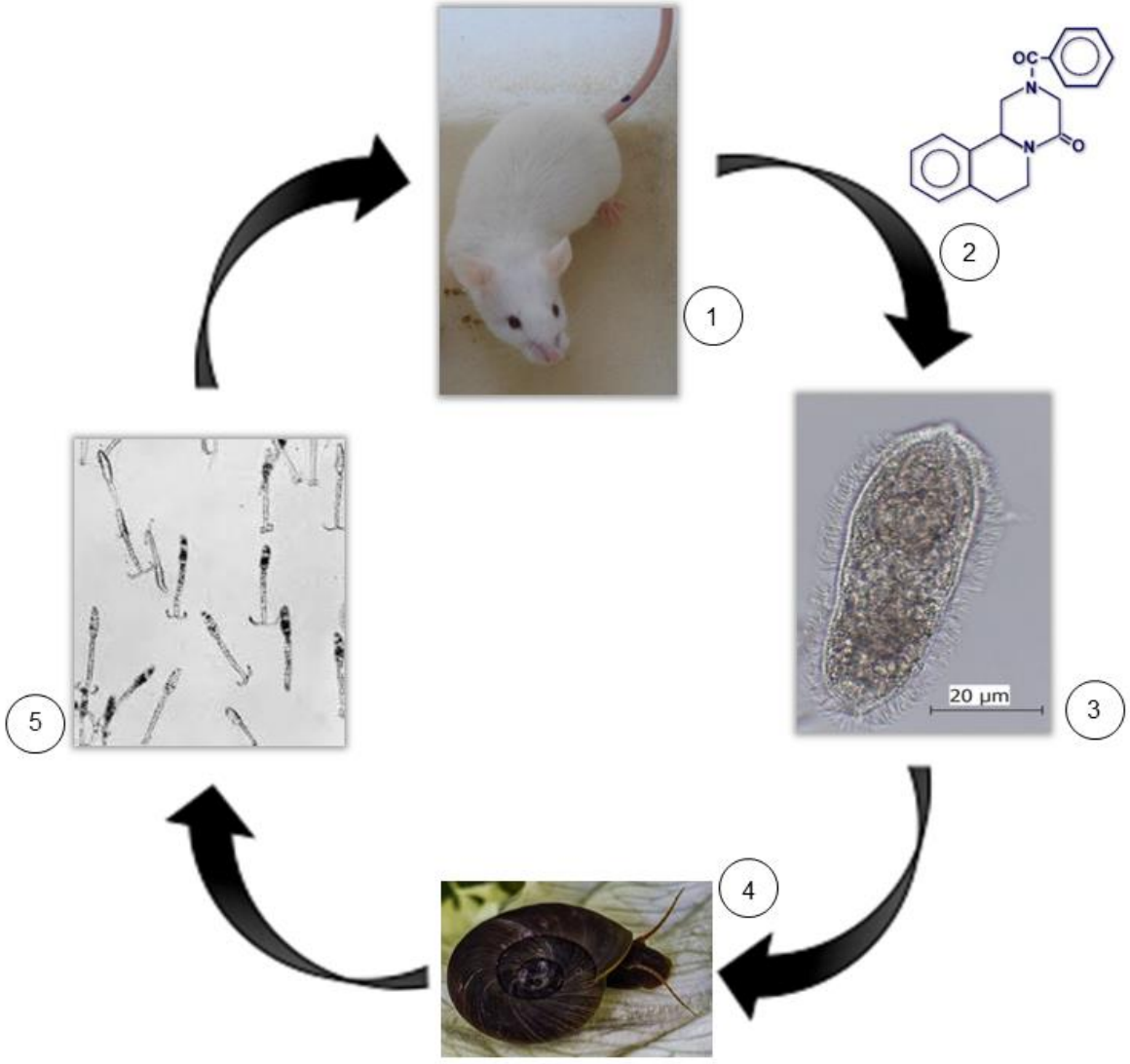

Figure II-1. Selection of *S. mansoni* PZQ-resistant strain. This selection was carried out under continuous PZQ increased pressure using CD1 mice over several passages. 1 - Transcutaneous infection of mice with ~100 cercariae; 2 - Oral administration of PZQ after infection confirmation by the presence of eggs in the feces (±60 days post-infection - dpi); 3 - Mice were euthanized to collect adult worms and miracidium (eggs in the liver) (±75 dpi); 4 - *B. glabrata* snails were infected with miracidium released from eggs; 5 - Cercariae were released from snails (±45 dpi).
doi:10.1371/journal.pone.0140147.g001.

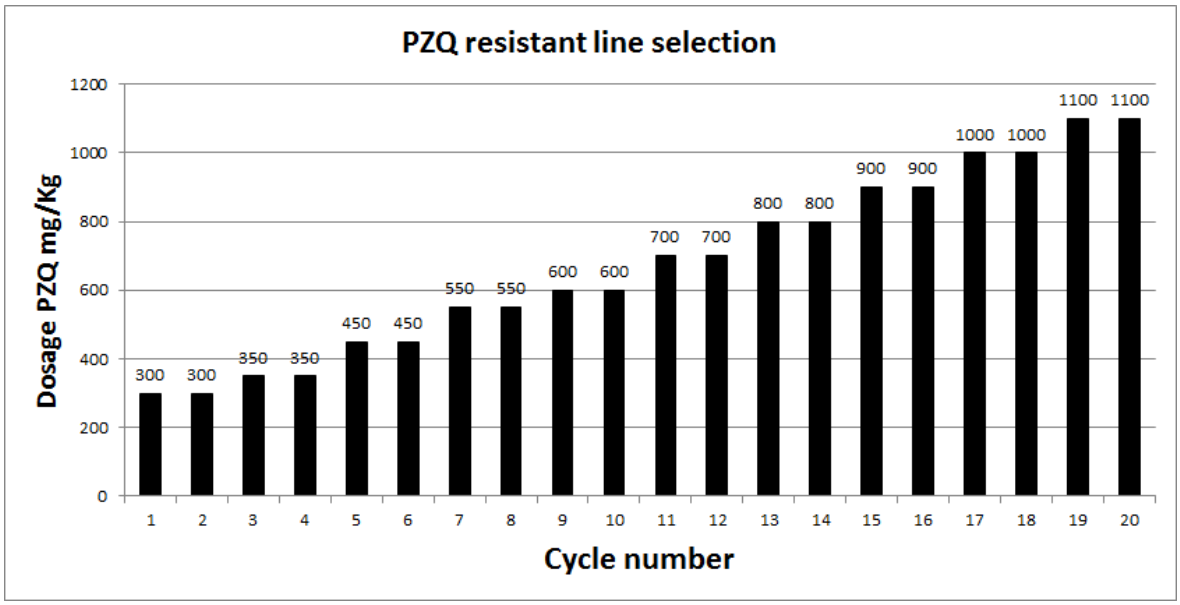

Figure II-2. **Schematic cartoon of PZQ doses during the selection procedure for the *S. mansoni* PZQ-resistant strain.** The parasite from BH susceptible strain was submitted to various steps of PZQ pressure, and the dose was increased along the cycle number of passages.
doi:10.1371/journal.pone.0140147.g002.

Adult worms (8–10 weeks post-infection) were collected by liver-perfusion, as described by Lewis and colleagues [24], and maintained in saline solution for the EtBr efflux assay or in RPMI medium (Sigma-Aldrich) for the *ex vivo* PZQ susceptibility assays.

3.4. Ethidium Bromide efflux assay

EtBr efflux assay was performed with the objective of comparing the EPs activity between males of both PZQ-susceptible and PZQ-resistant parasite strains as described by Viveiros and colleagues, adapted in this study for the assessment of parasite efflux activity [25]. Verapamil (2.2 μM and 4.4 μM), known as an inhibitor of ABCB1 (Pgp) efflux pump activity was used as EtBr efflux inhibitor at concentrations that did not compromise viability. EtBr concentration was previously optimized for each strain of adult worms in order to determine the lowest concentration which reflects the balance between EtBr accumulation by influx (passive diffusion) and extrusion by active efflux during the 35 minutes of the assay (EtBr influx-efflux steady-state whose accumulation - fluorescent signal - inside the worms is above the lowest signal detectable by the fluorescence microscope). This ensures that the observed increase of accumulation of EtBr during the 35 minutes of the assay is due to inhibition of EPs that promotes increased accumulation of the fluorophore inside the worms [25]. To

measure the time-curve of increased EtBr accumulation promoted by the inhibitor Verapamil, our EtBr control group were worms incubated with the same concentration of EtBr in the absence of Verapamil. All experiments were carried out in triplicate with three worms each (n = 9). For quantification of fluorescence, three areas, of each worm, of the same size, of the worm central section (below the cecum ramification), as shown in Figure II-3, were defined and fluorescence intensity was measured and quantified using ImageJ software (imagej.nih.gov) and background intensity was subtracted. Thus, each time-point of relative fluorescence in each assay corresponds to the mean of EtBr fluorescence (n = 9) that remains accumulated per unit of time that we compare to the EtBr control group (no inhibitor) [25].

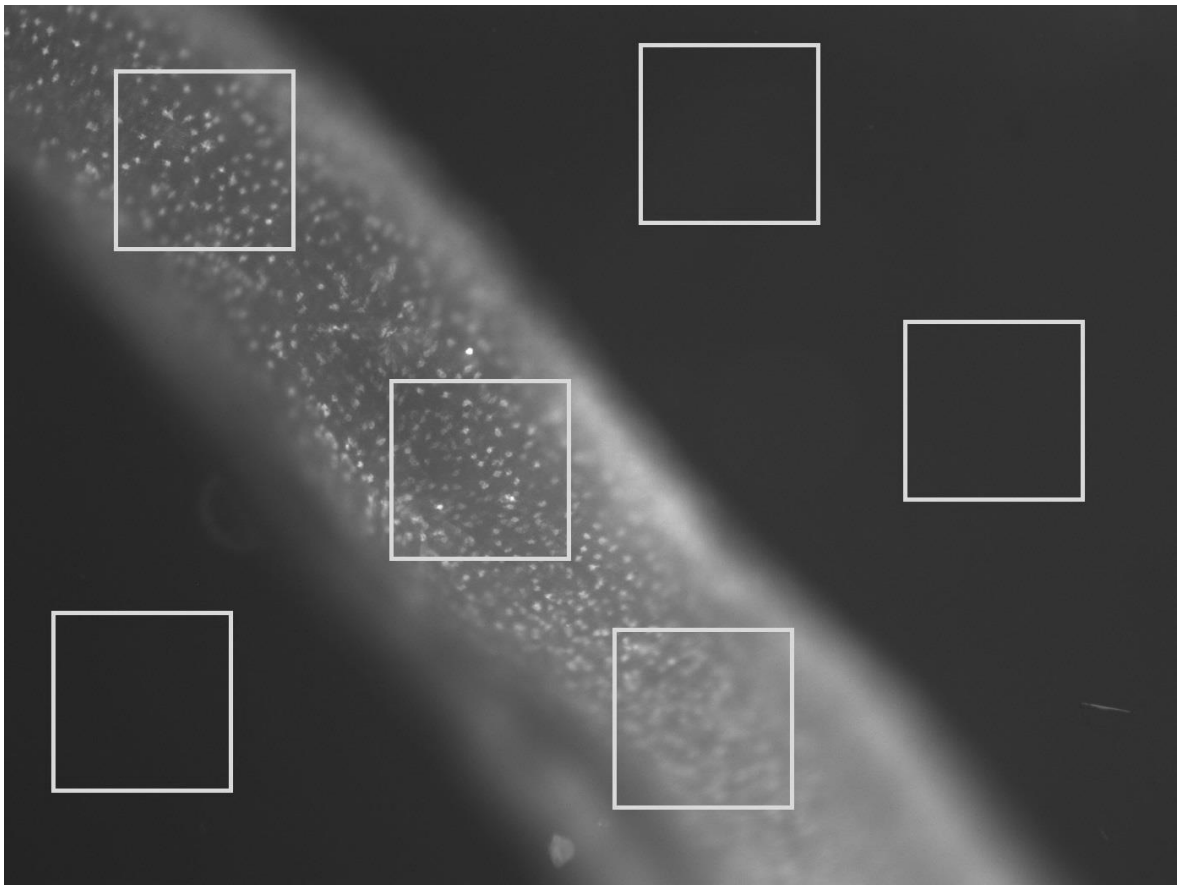

Figure II-3. Schematic representation of the worm areas analyzed by ImageJ. Fluorescence quantification was made in three defined regions, of the same size, corresponding to the worm central section (below the cecum ramification), of each worm and fluorescence intensity within each region was quantified using ImageJ software (imagej.nih.gov) and background intensity was subtracted. doi:10.1371/journal.pone.0140147.g003.

After collecting parasites (as described before), they were separated by sex, and only males were used for this experiment since EtBr binds non-specifically to the blood

present in the female's intestine, thus defaulting the experiment. 24-well culture plates were prepared using RPMI–1640 culture medium, 200 mM L-glutamine, 10 mM HEPES, 24 mM of $NaHCO_3$, 10,000 UI of Penicillin and 10 mg/mL of Streptomycin, from Sigma-Aldrich, pH 7 and supplemented with 15% fetal bovine serum and three parasites were added on each well for each studied group. Parasites were incubated overnight at 37 °C in a 5% CO_2 atmosphere to recover from stress caused by liver perfusion. After this period, the worms were washed twice with saline solution to clean any traces of culture medium. The worms were then exposed to the inhibitor for 1 h in the previous-mentioned concentrations, after which EtBr was added (0.6 µM) and parasites were observed under fluorescence microscopy (Zeiss, Axioskop HBO50) for a maximum of 35 min and pictures were taken every 2 min. After this period 1 mM of $CaCl_2$ was added to reverse the inhibitory effect of Verapamil, pictures were taken every two minutes for 35 min, of all worms at the same exact position, magnification and fluorescence intensity for overall analysis of the assays. Three control groups were used: 1- Without Verapamil, 2 - Without EtBr and 3 - Without both Verapamil and EtBr (negative control). Fluorescence was quantified using the ImageJ software and compared between different groups.

3.5. *Ex vivo* Praziquantel susceptibility assay

An *ex vivo* assay was devised to assess the susceptibility of adult worms of *S. mansoni* from both PZQ-susceptible and PZQ-resistant parasite strains, in the presence and absence of Verapamil, to ascertain the involvement of Pgp-like EPs in the PZQ resistant phenotype.

Parasites were collected as previously described and separated by sex. 24-well culture plates were prepared as described in the previous section and various concentrations of PZQ and Verapamil were used in this susceptibility assay (Table II-1). Five worms were kept in each well and the same concentration of drug and inhibitor was used in two wells of the same plate. The experiment was done in triplicate, with at least 30 worms used for each concentration of drug and inhibitor. After adding Verapamil and/or PZQ, parasites were incubated for another 24 h at 37 °C in a 5% CO_2 atmosphere after which the medium was switched for a drug free medium and kept for another 48 h. Parasites were observed every 12 h and the culture medium was changed after each observation. Parasites that did not present any movement after being observed at the

microscope for a period of 2 min were considered dead. Lethal doses were calculated using the software IBM SPSS Statistics software version 20.0 for Windows using Probit regression model with a 95% confidence. The lethal doses obtained were used for graphical construction design, using GraphPad Prism software.

Table II-1. PZQ and Verapamil concentrations used for the *ex vivo* PZQ susceptibility assay.

Parasite strains	Parasite Sex	Verapamil (μM)	PZQ (μM)
Susceptible	Males	0.0	0 - 25.6
		0.2	0 - 25.6
		1.1	0 - 25.6
	Females	0.0	0 - 288.1
		4.4	0 - 288.1
Resistant	Males	0.0	0 - 128.0
		1.1	0 - 64.0
		2.2	0 - 48.0
		4.4	0 - 48.0
		8.8	0 - 32.0
	Females	0.0	0 - 2880.9
		8.8	0 - 2880.9

doi:10.1371/journal.pone.0140147.t001

3.6. RNA extraction and real-time qRT-PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, California, USA) from quick-frozen worms and then treated with DNase from Ambion according to the manufacturer's instructions. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the PerfectaSYBR Green SuperMix for iQ from Quanta Biosciences on an Opticon Real-Time PCR detection system from BioRad, according to the manufacturer's recommendations. *Schistosoma mansoni 18S* (*Sm18s*) ribosomal RNA of each group was used as a reference gene in these experiments. Primers used for the amplification of *SmMDR2* gene were *SmMDR2* F (5'-TCTGACAATCGACCTGGTG–3') and *SmMDR2* R (5'-CCAAGGAAGCAATGACTAAAAC–3') and for *Sm18S* gene the primers were *Sm18S*

F (5'-AGGAATTGACGGAAGGGCAC–3') and *Sm18S* R 5'ACCACCCACCGAATCAAGAAAG–3') [21]. For quantitative measurements, data was analyzed using the $2^{-\Delta\Delta Ct}$ method [26] to determine the relative expression ratio between target gene (*SmMDR2*) and reference housekeeping gene (*Sm18S*), with appropriate calibrators and corrections for amplification efficiency. Three biological and technical replicates were used for the qRT-PCR experiments.

3.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD), and tested for statistical significance using either ANOVA or unpaired t-tests. Probit regression model with a 95% confidence was used to calculate the lethal doses, and the graphic construction was performed using GraphPad Prism 5.0 software.

3.8. Ethics statement

This research project was reviewed and approved by the Ethics Committee and Animal Welfare, Faculty of Veterinary Medicine, UL (Ref. 0421/2013). Animals were maintained and handled in accordance with National and European legislation (DL 276/2001 and DL 314/2003; 2010/63/EU adopted on 22nd September 2010), with regard to the protection and animal welfare, and all procedures were performed according to National and European legislation. The anesthetics and other techniques were used to reduce the pain and adverse effect of animal.

4. Results

4.1. Ethidium Bromide efflux assay

Efflux pump activity was compared between PZQ-resistant and PZQ-susceptible adult males through fluorescence microscopy observation. EtBr is a common substrate to all EPs, when outside the cells the signal is low, but when inside, the signal is amplified, and can be detected and quantified by time-course fluorescence spectroscopy [27]. Intracellular accumulation of EtBr after efflux inhibition by Verapamil was assessed by the increases in fluorescence intensity, using ImageJ software. As shown in Figure II-4 in the susceptible variant strain after exposure to 2.2 μM of Verapamil, the efflux of EtBr was inhibited resulting in a clear increase of fluorescence, which decreased after the addition of $CaCl_2$. Verapamil is known to block the flow of Calcium ions by binding to putative Ca^{2+} binding site [28–31], the addition of Calcium revealed a reversing effect on the Verapamil inhibitory action on the efflux of EtBr, apparently restoring the function of the adult worms EPs, thus reinforcing the hypothesis raised that the observed accumulation of EtBr in the adult males was due to the effect of this inhibitor on Calcium-dependent transporters, possibly by indirectly interfering with Calcium-dependent Pgp ATPases [28–31]. The control groups without EtBr showed viability and no intrinsic fluorescence was observed thus it is not represented. These findings are of importance considering that Pgp and MRPs are members of the “traffic ATPase” superfamily, which use the energy of ATP hydrolysis for maintaining their membrane transport function.

In the PZQ-resistant parasite strain, after exposure to 2.2 μM of Verapamil, there was an initial increase in fluorescence that later stabilized, showing EtBr accumulation levels lower than the PZQ-susceptible strain. A decrease in fluorescence was noticed after exposure to $CaCl_2$. Only by exposing the PZQ-resistant strain to 4.4 μM of Verapamil, fluorescence levels reached levels similar to the susceptible parasites (Figure II-5).

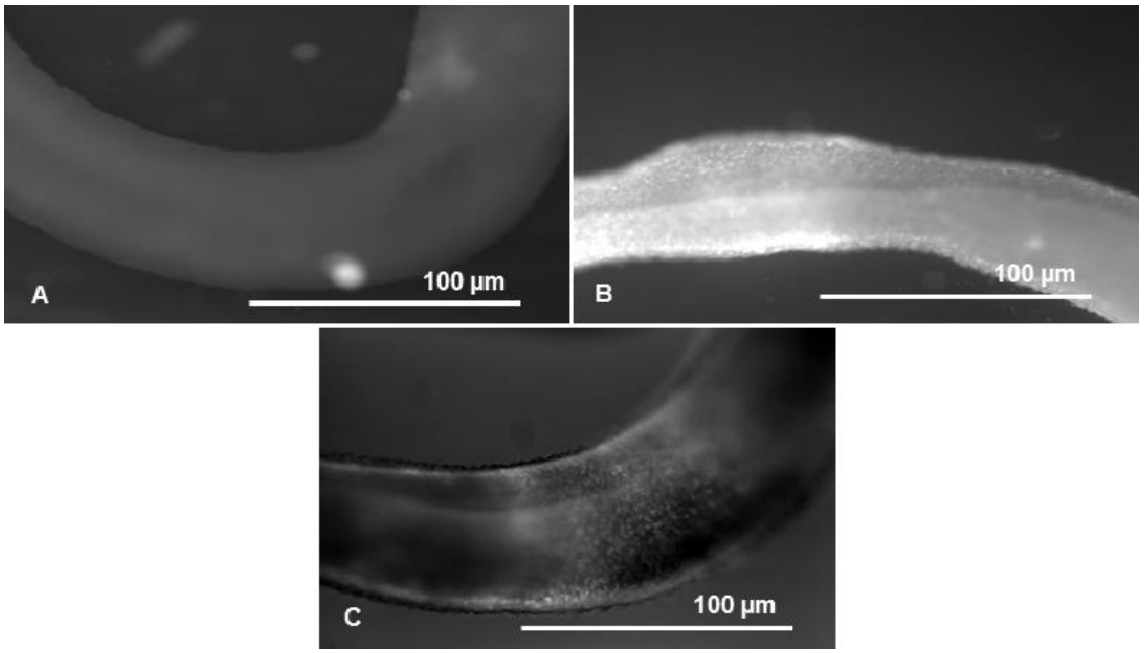

Figure II-4. EtBr efflux assay in adult males of *S. mansoni* PZQ-susceptible strain. A) Control group - worms exposed to 0.6 µM of EtBr (20 min); B) Worms exposed to 2.2 µM of Verapamil and 0.6 µM of EtBr (20 min); C) Worms exposed to 2.2 µM of Verapamil, 0.6 µM of EtBr, and 1 mM de $CaCl_2$ (35min). doi:10.1371/journal.pone.0140147.g004.

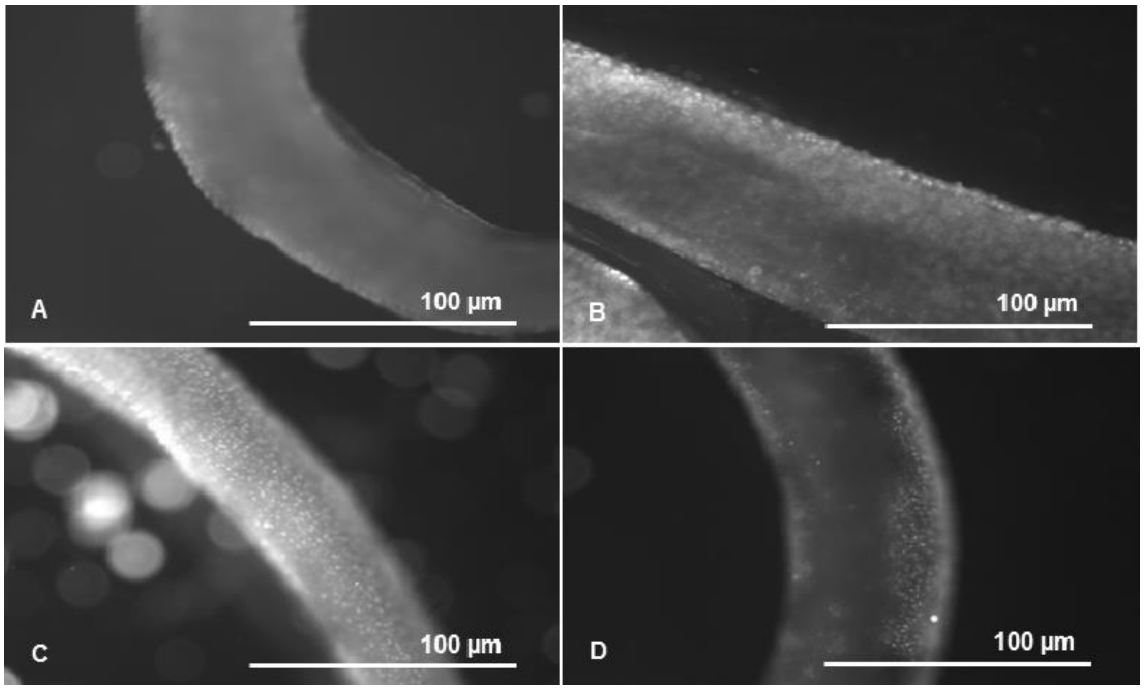

Figure II-5. EtBr efflux assay in adult males of *S. mansoni* PZQ-resistant strain. A) Control group - worms exposed to 0.6 µM of EtBr (20 min); B) Worms exposed to 2.2 µM of Verapamil and 0.6 µM of EtBr (20 min); C) Worms exposed to 4.4 µM of Verapamil and 0.6 µM of EtBr (20 min); D) Worms exposed to 4.4 µM of Verapamil, 0.6 µM of EtBr, and 1 mM de $CaCl_2$ (35 min). doi:10.1371/journal.pone.0140147.g005.

As described in the Material and Methods section of this Chapter, throughout the efflux assays, fluorescence microscopy images were taken every 2 min for 35 min. Fluorescence was quantified in each picture in three areas of the worm central section (below the cecum ramification), as shown in Figure II-3, and background fluorescence was subtracted for each parasite (n = 9) at each time-points. The average was calculated and real-time efflux graphics were created to obtain an EtBr accumulation time course in presence and absence of Verapamil in both variant strains (Figure II-6, Figure II-7 and Figure II-8). In the PZQ-susceptible parasite strain exposure of the worms to 2.2 μM of Verapamil allowed us to observe a steady increase in the fluorescence over time, reaching approximately twice the mean relative fluorescence levels after 20 min, once compared to parasites not exposed to Verapamil. After the addition of 1 mM $CaCl_2$ a sharp decrease in the fluorescence levels, reaching the same levels of those parasites not exposed to Verapamil (Figure II-6).

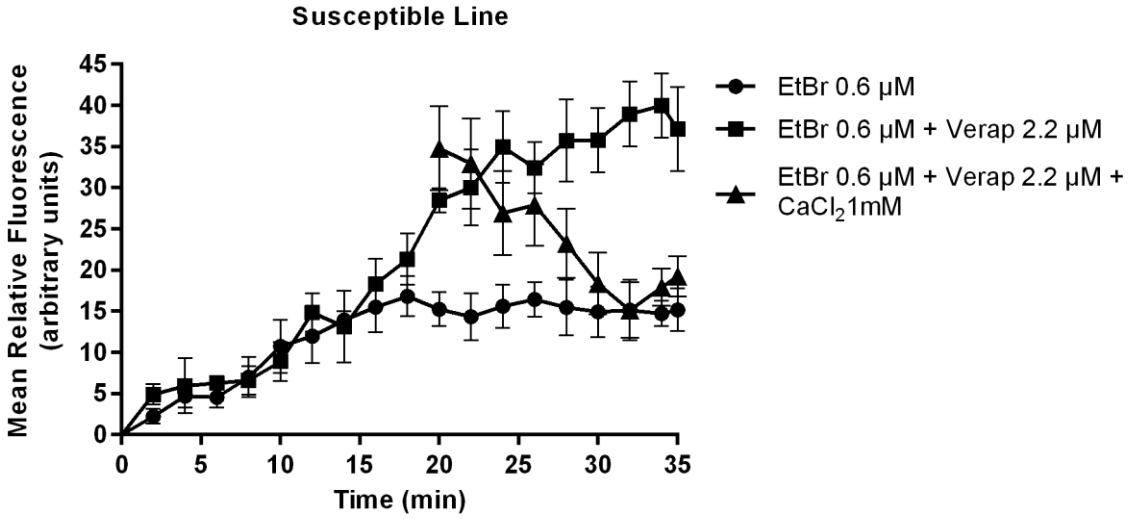

Figure II-6. Variation in EtBr accumulation (Mean relative fluorescence) in the presence and absence of Verapamil and after the addition of $CaCl_2$ in *S. mansoni* PZQ-susceptible adult males. Three worms were used for each group and the experiment was performed three times. Quantification measurements were made in three areas of the worm central section (below the cecum ramification) and background fluorescence was subtracted for each parasite at each time-point. The average measurement was calculated for each time-point. Data are expressed as mean fluorescence of the EtBr accumulated intracellularly over time.
doi:10.1371/journal.pone.0140147.g006

In the PZQ-resistant strain, for the parasites exposed to 2.2 μM of Verapamil, there was an increase in fluorescence in the first 16 min, then maintaining a constant fluorescence over time at lower levels than the susceptible strain. No decrease in fluorescence was observed upon addition of 1 mM $CaCl_2$ (Figure II-7). Once exposed to 4.4 μM of Verapamil, the parasites showed a steady increase in the mean fluorescence over time (Figure II-8). The PZQ-resistant parasite strain only showed fluorescence accumulation levels similar to the PZQ-susceptible strain when exposed to twice the concentration of Verapamil.

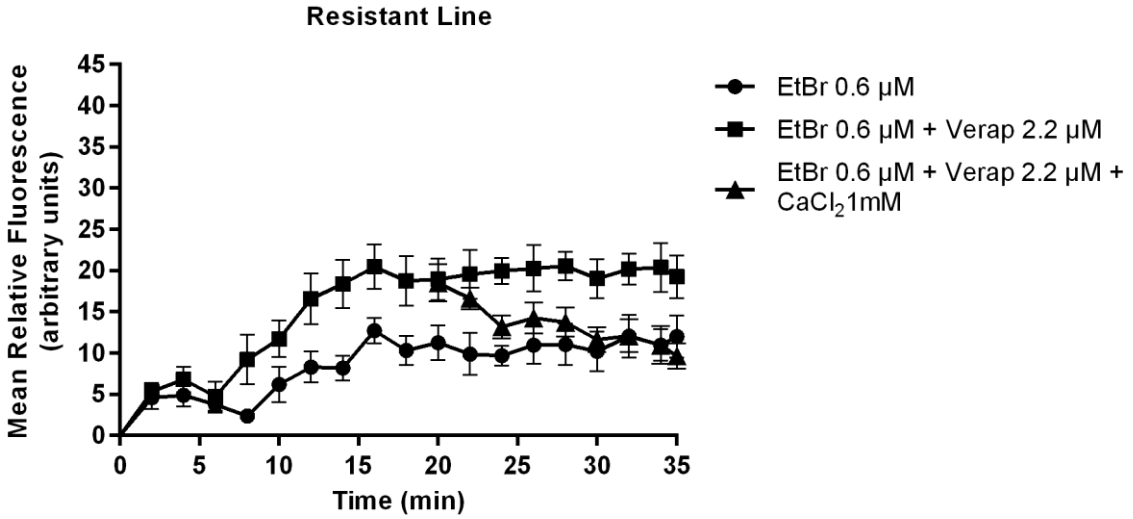

Figure II-7. Variation in EtBr accumulation (Mean relative fluorescence) in the presence and absence of Verapamil and after $CaCl_2$ addition in *S. mansoni* PZQ-resistant adult males. Three worms were used for each group and the experiment was performed three times. Quantification measurements were made in three areas of the worm central section (below the cecum ramification) and background fluorescence was subtracted for each parasite at each time-point. The average measurement was calculated for each time-point. Data are expressed as mean fluorescence of the EtBr accumulated intracellularly over time.
doi:10.1371/journal.pone.0140147.g007

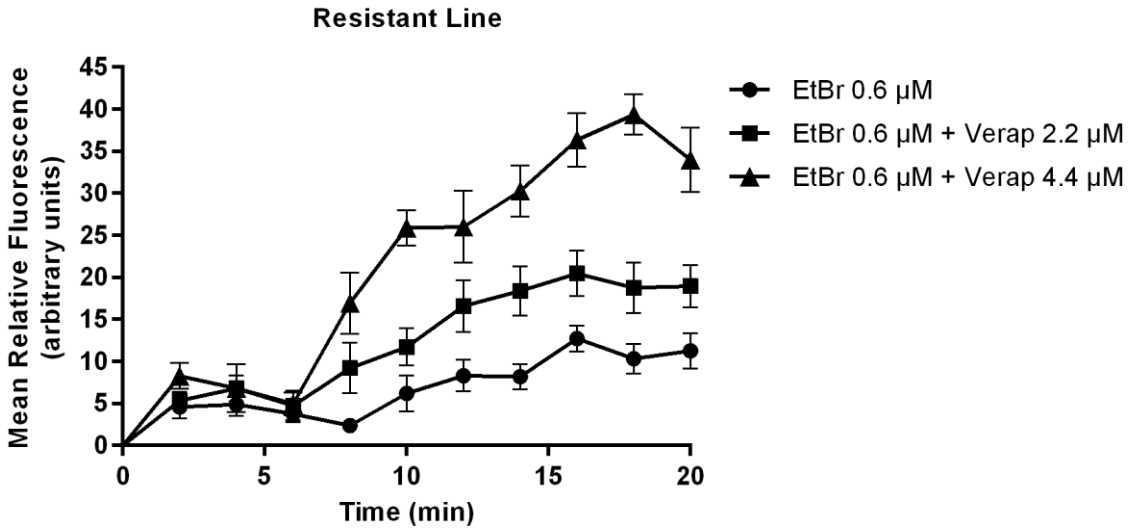

Figure II-8. Variation in EtBr accumulation (Mean relative fluorescence) in the absence and presence of 2.2 µM and 4.4 µM of Verapamil in *S. mansoni* PZQ-resistant adult males. Three worms were used for each group and the experiment was performed three times. Quantification measurements were made in three areas of the worm central section (below the cecum ramification) and background fluorescence was subtracted for each parasite at each time-point. The average measurement was calculated for each time-point. Data are expressed as mean fluorescence of EtBr accumulated intracellularly over time.
doi:10.1371/journal.pone.0140147.g008

4.2. *Ex vivo* Praziquantel susceptibility assay

PZQ-susceptible male worms. In the absence of Verapamil, adult males of the PZQ-susceptible strain achieved a 50% lethal dose (LD50) when exposed to 17.8 µM of PZQ; a lethal dose of 90% (LD90) when exposed to 24.2 µM of PZQ and a lethal dose of 99% (LD99) when exposed to 31.0 µM of PZQ. In the presence of Verapamil, it was possible to observe a reduction in the amount of PZQ required to achieve the lethal doses mentioned above. In the presence of 0.2 µM and 1.1 µM of Verapamil the LD50 was 15.1 µM and 12.5 µM of PZQ, respectively. LD90 was 20.6 µM and 16.9 µM of PZQ, and LD99 was 26.4 µM and 21.7 µM of PZQ, respectively (Table II-2).

Table II-2. Lethal doses of PZQ (LB - Lower bound; UB - Upper bound) calculated using Probit regression model with a 95% confidence, for *S. mansoni* PZQ-susceptible males in the presence of different concentrations of Verapamil.

Verapamil Concentration (μM)	Mortality (%)						
	1	10	30	50	70	90	99
	PZQ Concentration (μM)						
0	10.2 (LB – 8.37; UB – 11.21)	13.1 (LB – 10.53; UB – 15.00)	15.7 (LB – 13.41; UB – 17.70)	17.8 (LB – 15.62; UB – 20.15)	20.2 (LB – 17.89; UB – 23.34)	24.2 (LB – 21.21; UB – 29.58)	31.0 (LB – 26.14; UB – 40.11)
0.2	8.7 (LB – 6.11; UB – 10.53)	11.1 (LB – 8.73; UB – 12.94)	13.4 (LB – 11.16; UB – 15.21)	15.1 (LB – 13.06; UB – 17.24)	17.2 (LB – 15.05; UB – 19.83)	20.6 (LB – 17.99; UB – 24.83)	26.4 (LB – 22.32; UB – 35.26)
1.1	7.2 (LB – 5.06; UB – 8.66)	9.2 (LB – 7.22; UB – 10.64)	11.0 (LB – 9.52; UB – 12.52)	12.5 (LB – 10.78; UB – 14.21)	14.1 (LB – 12.41; UB – 16.37)	16.9 (LB – 14.82; UB – 20.60)	21.7 (LB – 18.26; UB – 29.37)

doi:10.1371/journal.pone.0140147.t002

The lethal dose (LD) values calculated using PZQ-susceptible parasite strain adult males were applied for the construction of mortality trend curves to get a better view of Verapamil effect on their susceptibility to PZQ (Figure II-9). A decrease in the PZQ concentration required to achieve the same level of mortality was evident once compared to parasites not exposed to the inhibitor.

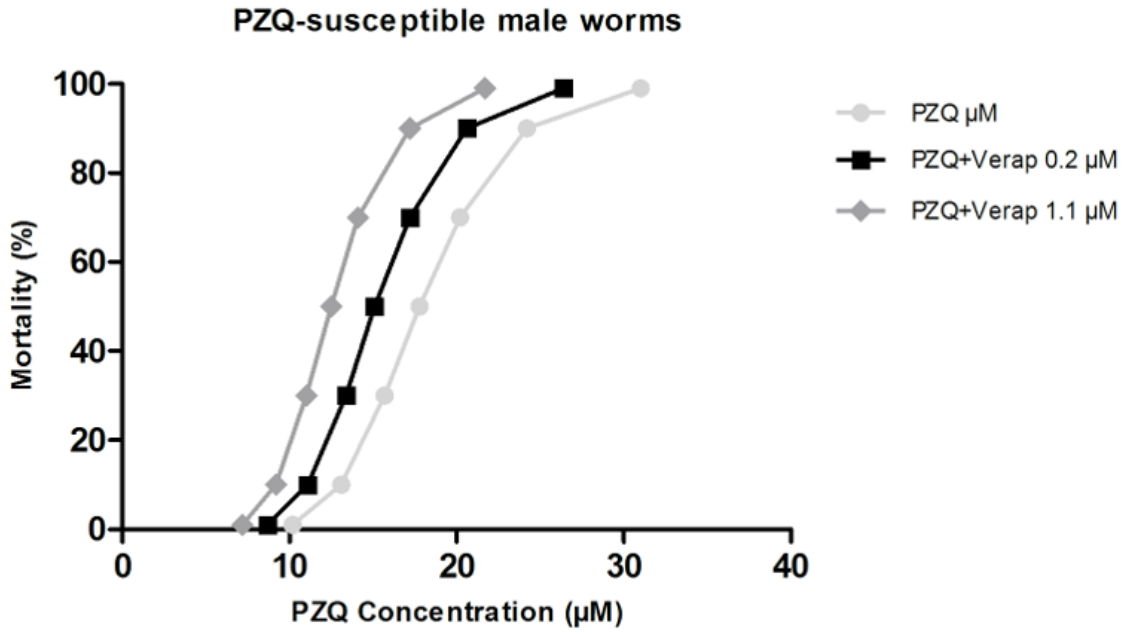

Figure II-9. Mortality trends of *S. mansoni* adult males PZQ-susceptible exposed to PZQ in the presence of Verapamil. The mortality levels to increase concentrations of Verapamil (0.2 and 1.1 μM) are represented by survival curves. Additionally, the survival curve of parasites unexposed to Verapamil is also represented. The Probit regression model was used with a 95% of confidence.
doi:10.1371/journal.pone.0140147.g009

PZQ-resistant male worms. In the absence of Verapamil, male worms of the PZQ-resistant strain achieved the LD50 when exposed to 65.2 μM of PZQ, LD90 when exposed to 98.1 μM and the LD99 when exposed to 137.0 μM of PZQ.

When exposed to a non-toxic concentration of Verapamil, it was possible to observe a reduction in the amount of PZQ required to achieve the lethal doses mentioned above. In the presence of four different concentrations of Verapamil (1.1 μM, 2.2 μM, 4.4 μM, and 8.8 μM), the PZQ lethal dose decreased significantly: LD50 concentrations of PZQ was 33.9 μM, 19.7 μM, 5.1 μM and 3.6 μM, LD90 was 52.4 μM, 37.5 μM, 19.8 μM and 12.8 μM and the LD99 was 74.7 μM, 63.2 μM, 59.8 μM and 35.9 μM, for each of the four concentrations of inhibitor used (Table II-3).

Table II-3. Lethal doses of PZQ (LB-Lower bound; UB-Upper bound) calculated using Probit regression model with a 95% confidence, for *S. mansoni* PZQ-resistant parasite strain males in the presence of various concentrations of Verapamil.

Verapamil Concentration (μM)	Mortality (%)						
	1	10	30	50	70	90	99
	PZQ Concentration (μM)						
0	30.98 (LB – 22.30; UB – 37.51)	43.27 (LB – 35.15; UB – 49.15)	55.11 (LB – 48.34; UB – 60.44)	65.16 (LB – 59.27; UB – 70.95)	77.05 (LB – 70.76; UB – 85.51)	98.14 (LB – 87.99; UB – 116.32)	137.03 (LB – 115.75; UB – 182.62)
1.1	15.39 (LB – 9.91; UB – 19.23)	21.95 (LB – 15.62; UB – 28.42)	28.38 (LB – 25.42; UB – 31.37)	33.92 (LB – 27.81; UB – 37.76)	40.53 (LB – 38.20; UB – 52.21)	52.41 (LB – 45.26; UB – 58.07)	74.74 (LB – 64.41; UB – 84.63)
2.2	6.16 (LB – 1.46; UB – 9.83)	10.38 (LB – 4.34; UB – 14.20)	15.17 (LB – 9.21; UB – 19.18)	19.72 (LB – 14.55; UB – 25.21)	25.64 (LB – 20.38; UB – 37.35)	37.46 (LB – 28.43; UB – 76.87)	63.20 (LB – 41.46; UB – 88.66)
4.4	0.44 (LB – 0.08; UB – 1.02)	1.33 (LB – 0.42; UB – 2.35)	2.95 (LB – 1.42; UB – 4.37)	5.13 (LB – 3.18; UB – 6.93)	8.92 (LB – 6.56; UB – 11.95)	19.85 (LB – 14.44; UB – 34.00)	59.81 (LB – 34.70; UB – 67.70)
8.8	0.36 (LB – 0.02; UB – 0.94)	1.01 (LB – 0.38; UB – 1.86)	2.15 (LB – 1.25; UB – 3.00)	3.60 (LB – 2.98; UB – 4.98)	6.05 (LB – 5.35; UB – 9.02)	12.79 (LB – 10.18; UB – 17.60)	35.94 (LB – 22.72; UB – 40.65)

doi:10.1371/journal.pone.0140147.t003

The lethal PZQ dose values for PZQ-resistant males when exposed to different concentrations of Verapamil were plotted in a mortality dose dependent curve (Figure II-10), showing the effect of Verapamil on the susceptibility to PZQ in this variant strain. Once again, in the presence of the efflux inhibitor (Verapamil), a decrease in the PZQ concentration required to achieve the same level of mortality was observed, compared

to parasites not exposed to this inhibitor. In the presence of 1.1 μM of Verapamil, the lowest concentration tested in this strain, the PZQ lethal concentrations were twice as low compared to the ones obtained for the group not exposed to the inhibitor. Overall, it was demonstrated that the drug-resistant strain reduces or reverts its resistance to PZQ in the presence of Verapamil obtaining LD values close to or even lower than those obtained for the susceptible variant strain.

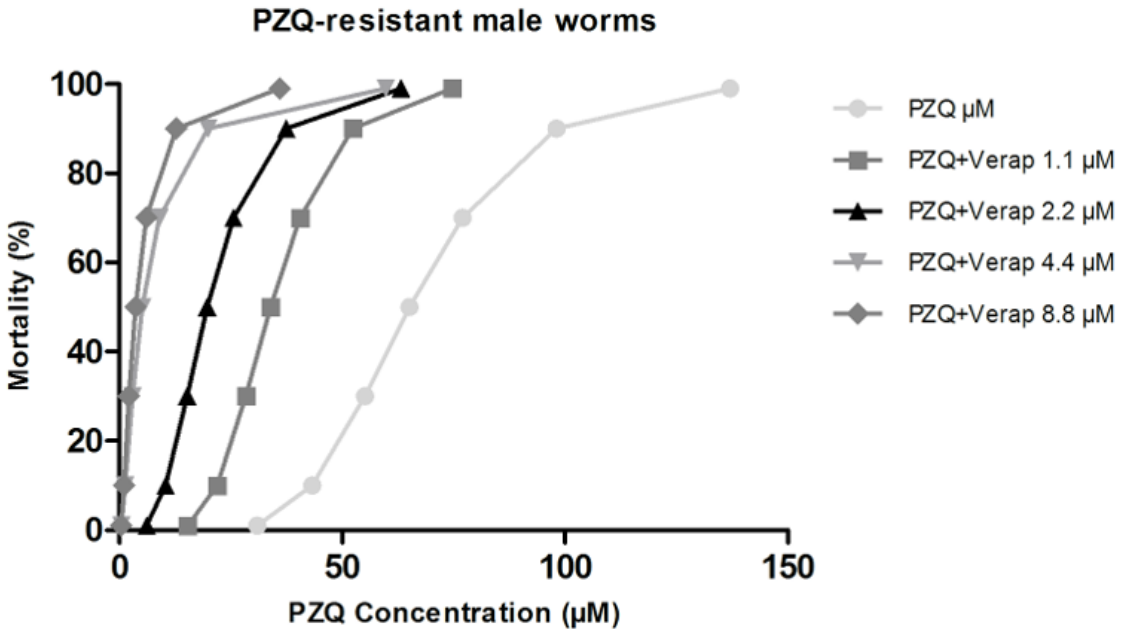

Figure II-10. Mortality trends of *S. mansoni* adult males PZQ-resistant exposed to PZQ in the presence of Verapamil. The mortality levels to increase concentrations of Verapamil (1.1–8.8 μM) are represented by survival curves. Additionally, the survival curve of parasites unexposed to Verapamil is also represented. The Probit regression model was used with a 95% of confidence.
doi:10.1371/journal.pone.0140147.g010

PZQ-susceptible female worms. In the absence of Verapamil, susceptible strain females presented a LD50 of 205.02 μM, a LD90 of 230.84 μM, and a LD99 of 254.29 μM of PZQ (Table II-4). When exposed to the highest concentration of Verapamil used in this study (4.4 μM), no differences in PZQ susceptibility were noticed (Figure II-11). Our results put in evidence that PZQ-susceptible female worms are more resistant to PZQ than males from the resistant strain.

Table II-4. Lethal doses of PZQ (LB—Lower bound; UB—Upper bound) calculated using Probit regression model with a 95% confidence, for *S. mansoni* PZQ-susceptible parasite strain females in the presence of different concentrations of Verapamil.

Verapamil Concentration (μM)	Mortality (%)						
	1	10	30	50	70	90	99
	PZQ Concentration (μM)						
0	165.29 (LB – 156.91; UB – 171.61)	182.08 (LB – 176.07; UB – 186.74)	195.30 (LB – 190.94; UB – 199.00)	205.02 (LB – 201.34; UB – 208.62)	215.21 (LB – 211.43; UB – 219.61)	230.84 (LB – 225.60; UB – 237.86)	254.29 (LB – 245.62; UB – 266.75)
4.4	159.22 (LB – 150.43; UB – 168.33)	178.33 (LB – 166.08; UB – 181.26)	193.52 (LB – 189.62; UB – 195.99)	204.76 (LB – 200.25; UB – 208.55)	216.62 (LB – 209.86; UB – 219.01)	234.90 (LB – 230.22; UB – 238.73)	252.98 (LB – 244.44; UB – 263.93)

doi:10.1371/journal.pone.0140147.t004

Although the concentration of PZQ associated with female worm data seems high, PZQ used *ex vivo* is not metabolized, and PZQ metabolites have shown a higher anti-schistosomal activity than pure unmetabolized PZQ (used *in vitro*) [32].

It should also be noticed that *Schistosoma* females have a much higher tolerance to PZQ than males as shown by Pica-Mattoccia and Cioli [33] and by Liang and colleagues [34]. The results we obtained for LD50 in females stays somewhere in between the ones obtained by these authors.

PZQ-resistant female worms. PZQ-resistant female worms were exposed to PZQ concentrations up to 2880.92 μM, in the presence and absence of Verapamil, and it was not possible to determine any lethal doses. However, long-term effects of PZQ and the effects of the host immune system were not taken into account.

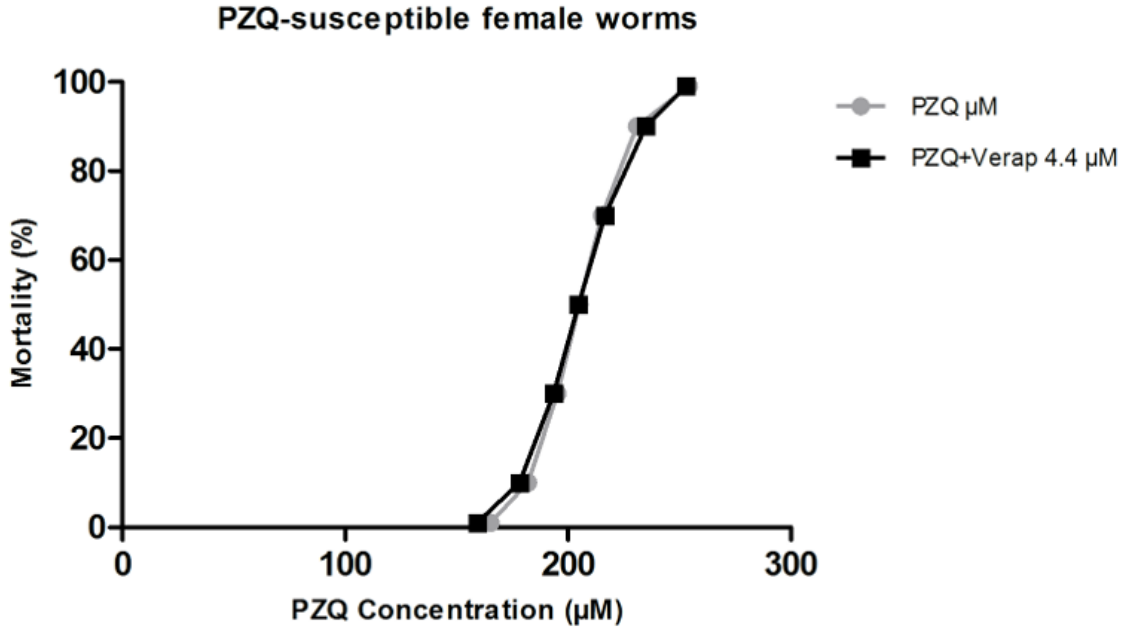

Figure II-11. Mortality trends *S. mansoni* adult females PZQ-susceptible exposed to PZQ in the presence of Verapamil. The mortality levels to 4.4 µM Verapamil is represented by a survival curve. Additionally, the survival curve of parasites unexposed to Verapamil is also represented. The Probit regression model was used with a 95% of confidence.
doi:10.1371/journal.pone.0140147.g011

4.3. Real-time qRT-PCR

The relative expression levels of *SmMDR2* gene were assessed using a qRT-PCR method. Adult worms of each PZQ-strain were separated by sex and compared. Parasites were also compared in the presence and absence of 0.3 µM of PZQ for 3 h. As shown in Figure II-12, when comparing PZQ-susceptible males and females, before exposure to PZQ, females showed a relative increase in the expression level of *SmMDR2* of approximately 4 times, when compared to males ($p < 0.05$). When exposed to PZQ the expression level of *SmMDR2* in susceptible males increased 17 times when compared to the expression level of the same gene in the absence of PZQ in females ($p < 0.05$). As expected, PZQ-resistant males showed, in the absence of PZQ, an increase in the expression level of *SmMDR2* of approximately 32 times when compared to PZQ-susceptible males ($p < 0.05$). Furthermore, after exposure to PZQ, *SmMDR2* expression level of PZQ-resistant males was approximately 6 times higher than in the absence of PZQ ($p < 0.05$). Finally, PZQ-resistant females showed no significant change in *SmMDR2* expression after exposure to PZQ ($p > 0.05$), and the

expression was approximately 10 times lower than the PZQ-susceptible females ($p < 0.05$).

In the EtBr efflux assay, when observing both PZQ-susceptible and PZQ-resistant adult males, fluorescence levels in the absence of Verapamil, did not vary significantly, however when observing the expression of *SmMDR2* through qRT-PCR, there was a significant difference in *SmMDR2* expression. However we believe this could be explained by a higher sensitivity of qRT-PCR.

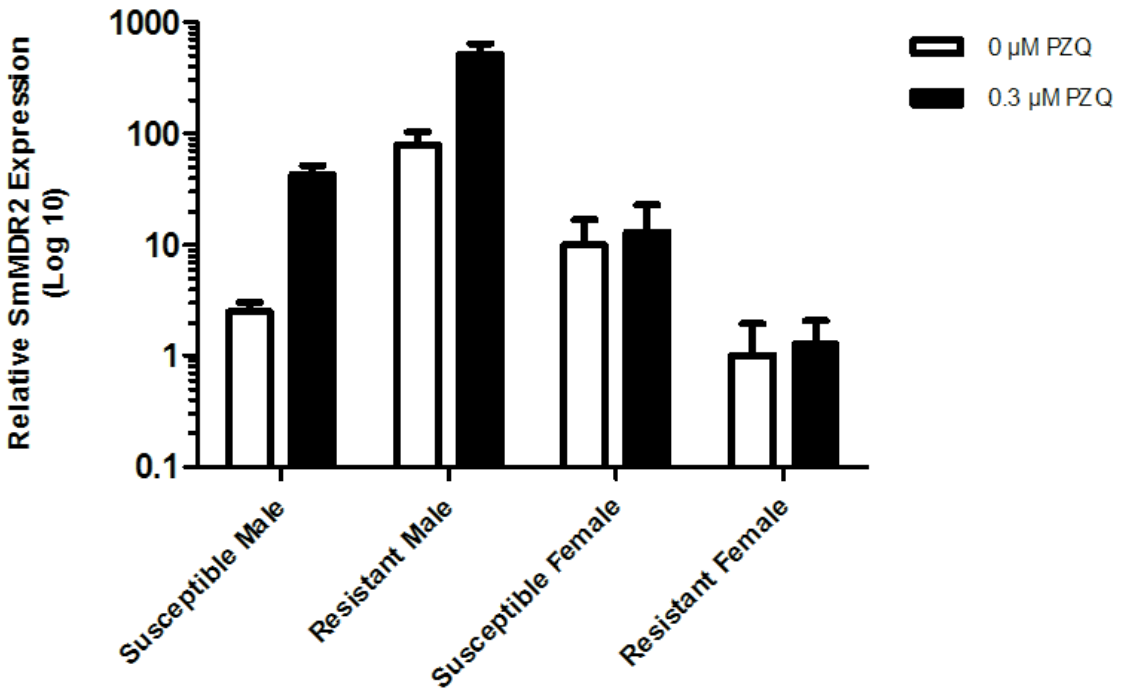

Figure II-12. Relative expression level of *SmMDR2* in males and females of PZQ-susceptible and PZQ-resistant parasite strains in the presence and absence of PZQ. White bars - level expression of *SmMDR2* in adult worms without exposure to PZQ, and black bars - level expression of *SmMDR2* in adult worms after exposure to PZQ. The n-fold changes were determined by qRT-PCR using *S. mansoni 18S* (*Sm18s*) of each group as a reference gene. Differences of the relative level of *SmMDR2* between the groups was done using ANOVA and unpaired t-test, $p < 0.05$.
doi:10.1371/journal.pone.0140147.g012

5. Discussion

We have selected, for the first time, to our knowledge, a stable PZQ-resistant parasite strain that resists to 1,200 mg/kg of PZQ. This strain was obtained by PZQ continuous drug pressure, and this PZQ-resistant parasite strain was used to analyze the involvement of EPs in the observed induced PZQ-drug resistance phenotype. EPs activity of *S. mansoni* adult male worms, was observed and monitored by fluorescence microscopy using for the first time an adaptation of the semi-automated fluorometric methodology described by Viveiros and colleagues [25]. EtBr was used as a universal fluorescent substrate in the presence and absence of an efflux inhibitor – Verapamil, thus the emission of the accumulated fluorescence was monitored throughout sequential photographs, taken every 2 min, during a maximum period of 35 min. This assay was only possible to perform with adult males, because EtBr binds non-specifically to the blood present in the female's intestine, turning impossible to distinguish differences of EPs activity in the female worms [35].

In PZQ-susceptible adult males, the exposure to 2.2 μM of Verapamil led to a substantial increase in accumulated fluorescence suggesting that Verapamil is able to inhibit EtBr efflux in *S. mansoni* males of the susceptible strain. Reversal of this effect was possible after the addition of a non-toxic concentration of $CaCl_2$, suggesting that $CaCl_2$ has an important role in the mechanism responsible for reversing the efflux inhibitory effect of Verapamil in *Schistosoma* spp. This involvement of Calcium ions has been previously described in the literature [28–31] with a possible relation between Calcium homeostasis and Pgp mediated MDR reported by Sulová and colleagues [30]. At present, there is no scientifically accepted mechanism by which Calcium reverses the effects of Verapamil on Pgp activity, but our novel observation raises questions that will be explored in future works, namely the connection between Calcium homeostasis, Pgp activity and energy/ATP synthesis used for active transport of substrates [29, 30]. In PZQ-resistant adult males EtBr accumulation was up to 2 times lower than the PZQ-susceptible males, when exposed to 2.2 μM of Verapamil. Only when PZQ-resistant adult males were exposed to 4.4 μM of Verapamil the intracellular accumulation of EtBr was similar to the susceptible variant strain. This suggests that males of the resistant strain have a higher number of transporters responsible for the EtBr efflux, which was further demonstrated by the qRT-PCR results on the *SmMDR2* expression level. Other authors have also shown an increased expression level of the

SmMDR in PZQ-resistant clinical isolates of *S. mansoni* [19, 21, 22, 36, 37]. Here in this work, we were able to demonstrate that there is an increase of Pgp-like EPs activity in male worms from resistant strain which is in agreement with our results obtained by qRT-PCR for parasites exposed for 3 h with sub lethal concentrations of PZQ and correlation to its reduced susceptibility to PZQ. Messerli and colleagues [22] observed an increase of *SmMDR2* mRNA in females after being exposed to PZQ for 24–48 h, which were not coincident with expression of *SmMDR2* protein. Despite the absence of the assay of *SmMDR2* expression at protein level our *ex vivo* PZQ susceptibility assay suggests that Pgp-like proteins do not play a relevant role on PZQ-susceptibility in female worms.

The greatest advantage of our experimental model over other PZQ-resistant parasites described in the literature is the fact that they are isogenic allowing comparing the influence of EPs in PZQ resistant phenotype within the same genetic background. Therefore, it was possible to observe that the *S. mansoni* adult males variant resistant to PZQ presented an increased efflux pump activity suggesting that Pgp-like EPs play an important role in PZQ-drug resistance in *S. mansoni*. In the EtBr efflux assay, when observing both PZQ-susceptible and PZQ-resistant adult males, fluorescence levels in the absence of Verapamil, resistant strain males showed lower levels of fluorescence. This could be explained by a higher number of EtBr EPs in the resistant strain, which, is further reinforced when observing the expression of *SmMDR2*, through qRT-PCR, where a significant difference in *SmMDR2* expression can be observed. To further put in evidence that over-expression of EPs is involved in PZQ acquired drug-resistance, an *ex vivo* assay, using both *S. mansoni* strains, was performed to assess the degree of susceptibility of the adult parasites to PZQ, in the presence and absence of Verapamil. When adult males of susceptible strain were exposed to Verapamil the PZQ concentration required to reach lethal doses was lower than those observed in the absence of the inhibitor. Other authors have already reported that blocking the activity of the Pgp and MRPs transporters by Verapamil increases the pharmacological susceptibility of helminths such as *Caenorhabditis elegans*, *Haemonchus contortus*, and *Cooperia oncophora* to various anthelminthic drugs [38, 39]. For male worms of PZQ-resistant strain, in the presence of this efflux inhibitor, a lower PZQ concentration was required to achieve the same level of mortality compared to the same parasites not exposed to the inhibitor. In the presence of the lowest concentration of Verapamil

tested in the resistant strain, PZQ lethal concentrations were twice as low as the ones obtained for the group not exposed to the inhibitor.

Overall, it was possible to observe that PZQ susceptibility of the PZQ-resistant strain, in the presence of Verapamil, has LD values close to or even lower than those obtained for the PZQ-susceptible strain. Ardelli and Prichard also showed that a *C. elegans* Ivermectin-resistant strain in the presence of Verapamil, presented an increased susceptibility to Ivermectin, suggesting an involvement of Pgp-like EPs on this Ivermectin drug resistant phenotype [38]. Our results also suggest that, just as in the resistant strain of *C. elegans*, the adult males of our resistant strain have Pgp pumps involved in the drug resistance phenotype as demonstrated by the *SmMDR2* expression level analysis.

It is reported in literature collateral sensitivity of drug-resistant cancer cells to Verapamil [40–42], a phenomenon that might have happened in our *ex vivo* PZQ susceptibility assay in PZQ-resistant worms by a mechanism possibly linked to the expression of *SmMDR2*. This weakness observed by PZQ LD50 obtained from resistant worms in the presence of increasing concentrations of Verapamil, can circumvent potential problems that might be associated with adjuvant therapy using EPs inhibitors during standard therapy with PZQ, where the main objective is to treat patients by killing all the worms (susceptible and resistant worms) without causing side effects. Also, collateral sensitivity opens a new approach for the identification of new re-sensitizing compounds in the management of PZQ-resistance and to elucidate the mechanisms involved.

In contrast, female adult worms did not present any difference in the observed lethal doses of PZQ, in the presence or in absence of Verapamil, which gives an indication that the activity of Pgp-like EPs is not involved in PZQ susceptibility of adult female worms. Furthermore, the values obtained for lethal dose suggests a higher tolerance of female worms to PZQ. This higher tolerance has already been described in other reports [21, 33, 36] in which adult female worms tolerate considerably higher concentrations of PZQ than adult males both *ex vivo* and *in vivo* [9, 11, 12, 43, 44, 45].

Previous studies regarding drug resistance, have already presented evidence of an increased tolerance to PZQ in male worms [19, 21, 22, 36, 37, 46]. It should be noted that when using *in vitro* and *ex vivo* assays the interaction between the effects caused

by the drug and those caused by the host immune system on the parasite are not taken in consideration [33, 47, 48]. In conclusion, our work describes for the first time, the application of a successful methodology previously applied in bacteria and cancer cells, using the universal efflux pump substrate EtBr, for the evaluation of drug transporter systems on *S. mansoni* adult worms as a multicellular cell model using an *ex vivo* assay. The methodology used have demonstrated the involvement of adult male schistosomes Pgp-like transporters *SmMDR2* in PZQ drug resistance phenotype, evidenced by the fact that lower doses of Verapamil successfully reverted PZQ drug resistance when using sub lethal concentrations of PZQ. World Health Organization warns about the possible emergence of *Schistosoma* spp. populations that are resistant to PZQ, thus recommending continued vigilance [49]. Therefore, studies on genetic resistance mechanisms against PZQ are of extreme importance to understand the potential mechanism(s) of resistance/increased tolerance to PZQ, contributing to the development of new drugs and the delineation of new strategies for schistosomiasis control.

6. References

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CHAPTER III – RESEARCH WORK 2

III. Praziquantel-resistance in *S. mansoni*: morphological analysis of resistant and susceptible strains

Adapted from: Pinto-Almeida, A., Mendes, T., de Oliveira, R.N., Corrêa, S.A.P., Allegretti, S.M., Belo, S., Tomás, A., Anibal, F.F., Carrilho, E., and Afonso, A. Morphological characteristics of *Schistosoma mansoni* PZQ-resistant and -susceptible strains are different in presence of Praziquantel. *Front Microbiol.* 7:594. doi: 10.3389/fmicb.2016.00594.

1. Abstract

Schistosomiasis is one of the most common human parasitic diseases whose socioeconomic impact is only surpassed by malaria. PZQ is the only drug commercially available for the treatment of all schistosome species causing disease in humans. However, there are strong evidences of PZQ-resistance on *S. mansoni* and thus it is very important to study the phenotypic characteristics associated with it. The aim of this study was to evaluate morphological alterations in *S. mansoni* PZQ-resistant adult worms and eggs, by comparing a PZQ-resistant strain obtained under PZQ drug pressure with a PZQ-susceptible strain. For this, SEM was used to assess tegumental responsiveness of both strains under PZQ exposure, and optical microscopy allowed the monitoring of worms and eggs in the presence of the drug. Those assays showed that PZQ-susceptible worms exposed to the drug had more severe tegumental damages than the resistant one, which had only minor alterations. Moreover, contrary to what occurred in the susceptible strain, resistant worms were viable after PZQ exposure and gradually regaining full motility after removal of the drug. Eggs from resistant strain parasites are considerably smaller than those from susceptible strain. Our results suggest that there might be a difference in the tegument composition of the resistant strain and that those worms are less responsive to PZQ. Changes observed in egg morphology might imply alterations in the biology of schistosomes associated to PZQ-resistance, which could have impact on transmission and pathology of the disease. Moreover, we propose a hypothetical scenario where there is a different egg tropism of the *S. mansoni* resistant strain. This study is the first comparing the morphology of two strains that only differ in their resistance characteristics, which makes it a relevant step in the search for resistance determinants.

2. Introduction

Schistosomiasis is considered one of the most common human parasitic diseases, registering high rates of morbidity in about 20 million people, and about 280 000 deaths annually, especially in tropical and subtropical countries, namely African countries, countries of the Middle East, Caribbean, Brazil, Venezuela, Suriname, and other countries such as China, Indonesia and the Philippines [1-4]. The disease transmission invariably occurs when people suffering from schistosomiasis contaminate freshwater with their excreta containing parasite eggs, which hatch in water. People become infected when larval forms of the parasite (cercariae) penetrate the skin during contact with freshwater, usually by swimming or washing [5].

Schistosome infections can cause severe damage to various organs, mostly intestine, bladder, liver, brain and spinal cord, and cause significant morbidity, impair childhood development and adult productivity, potentially increase susceptibility to other infections such as HIV, and, in some cases, lead to death [1, 6-10]. Infrastructural and educational awareness can be highly effective to control schistosomiasis, however they are expensive and require levels of organization that are difficult in most developing countries [11]. The use of molluscicides to eliminate intermediate host snails is another important control method, but it is also costly and often produces limited and short-term effectiveness [12], as well as may have some negative environmental impacts. Therefore, because there is no available vaccine or prophylaxis, current control of schistosomiasis is based only on chemotherapy using PZQ [13].

PZQ is the only antischistosomal drug commercially available for the treatment of all human schistosome species [11, 14-16] and it presents important advantages such as mild side effects and relatively low cost [9]. PZQ has been available for several decades and large-scale PZQ treatment programs have produced significant reductions in both disease prevalence and intensity [17-19]. However, dependence on a single drug, which would be inadvisable for any infectious condition, is in the case of a disease with such high prevalence as schistosomiasis [20] a major concern, because it might induce the appearance of drug-resistant/tolerant parasites [21-25].

So far, the mechanism involved in the phenomenon of resistance to PZQ is not yet fully understood, there is only descriptions and evidences of this phenomenon in *in*

vivo and *ex vivo* studies. For instance, Fallon and Doenhoff [26], produced a *S. mansoni* PZQ-resistant strain in only two generations after repeated exposure to sub-lethal doses of the drug through *in vivo* artificial selection in mice. Furthermore, low cure rates in response to PZQ emerged 10-15 years ago after mass scale use in countries like Egypt and Senegal [27, 28] and worms from non-cured patients were repeatedly less susceptible to PZQ when tested in mice [29].

De Oliveira and colleagues [30], evaluated the effect of PZQ on the morphology of adult *S. mansoni* susceptible to PZQ and observed that parasites exposed to the drug showed tegumental changes apparent in all male and female worms. They observed destruction of tubercles with loss of thorns and formation of vesicles around the tubercles. Since the tegument of adult *Schistosoma* is a protective sheath that plays a role in defense, as well as in the uptake of nutrients, osmoregulation and excretion, damages in this structure may have major consequences to parasite viability. Thus, morphological studies are important to clarify aspects of drug-induced damages [31].

We have obtained in our laboratory by stepwise drug pressure a PZQ-resistant parasite strain (IHMT/UNL) from the fully PZQ-susceptible parasite BH strain. This *S. mansoni* variant strain is more resistant to PZQ than the original susceptible one and this resistant phenotype is stable in the absence of the drug pressure. This resistant parasite variant strain, obtained from infected mice, tolerates up to 1,200 mg PZQ/kg of mouse body weight and is isogenic to its fully susceptible parental counterpart, except for the genetic determinants accounting for the PZQ-resistance phenotype [32]. Besides that, data resulting from earlier study [32] suggested that the *S. mansoni* PZQ-resistant strain has different *ex vivo* susceptibility to PZQ and that this difference varied greatly between male and female worms. In this context, the objective of this study was to evaluate morphological alterations in the *S. mansoni* PZQ-resistance phenotype by comparing the PZQ-resistant strain obtained under PZQ drug pressure with the PZQ-susceptible strain.

3. Material and Methods

3.1. Praziquantel

PZQ was purchased from Merck & Co. (Kenilworth, NJ, USA) and dissolved in 1% DMSO from Sigma-Aldrich according to [33], used for stock solution, which was subsequently diluted to appropriate concentrations in culture media.

3.2. Parasite isolation and animal model

In this study we used two parasite strains, the *S. mansoni* BH strain, susceptible to PZQ, and a stable PZQ-resistant strain (IHMT/UNL) obtained from the BH strain as described in the Chapter II [32]. Briefly, our stable PZQ-resistant parasite strain was obtained from the BH line submitted to various steps of PZQ continuous drug pressure, starting with a sub therapeutic dose and finishing with 1,200 mg/kg of PZQ. Infected CD1 mice were checked approximately 60 days post parasite infection by Kato-Katz procedure. If eggs were found in feces, mice were then treated orally with PZQ solution at appropriate dose. If, on day 15, post PZQ treatment, viable eggs (verified by live miracidium inside the eggs and Kato-Katz procedure) continued to be eliminated, mice were euthanized and eggs present in the liver were used to obtain miracidium to subsequently infect *B. glabrata* snails. Once *B. glabrata* snails start eliminating *S. mansoni* cercariae (30 to 60 days after snail infection), new CD1 mice were re-infected and the previous procedure was repeated, continuing the PZQ-resistant strain selection *in vivo*. PZQ dose was increased every two passages. These two parasite strains are routinely kept in their intermediate host *B. glabrata* snails at our laboratory at IHMT/UNL.

Mus musculus CD1 line male mice are considered the choice animal model for *S. mansoni* infection, because it is highly susceptible to this parasite as it closely resembles the *S. mansoni* human infection [34]. The infection occurred by percutaneous exposure of mice tails to about 100 cercariae of *S. mansoni* each, through natural transdermal penetration of the cercariae [35].

Adult worms (8-10 weeks post-infection) were collected through hepatic portal system and mesenteric veins perfusion, according to [35], washed in saline solution and then maintained in a RPMI medium (Sigma-Aldrich). The Figure III-1 explains in more detail the experimental design performed in this chapter.

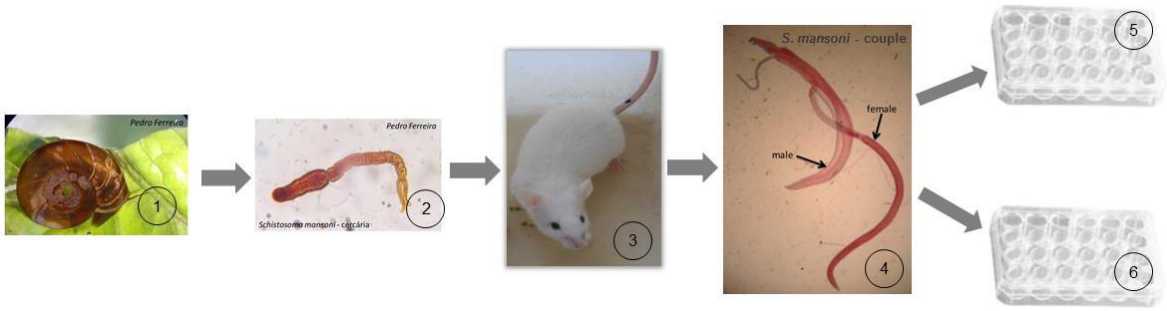

Figure III-1. Schematic cartoon of the experimental design. (1) *B. glabrata* snails (intermediate hosts of *S. mansoni*) release the infective form of the parasite (cercariae) for human or other mammalian definitive hosts; (2) About 100 cercariae were used to infect the definitive host; (3) CD1 Mice were used as definitive host in our experiment, and after 8-10 weeks post-infection they were sacrificed to collect adult worms of the parasite; (4) Adult worms were obtained by mice liver perfusion; (5) Male and female worms were treated in 24-well culture plate with a dose of PZQ (0.3 μM) with impact in the parasite but with the guarantee of not killing them. These worms were prepared for PZQ-induced tegumental alterations study using SEM; (6) Couple worms were treated in 24-well culture plate with a lethal dose of PZQ (32 μM). These worms were analyzed and monitored under an inverted optical microscope. The *B. glabrata,* cercariae and adult worms' photos were offered by Dr. Pedro Ferreira (IHMT/UNL).

3.3. *Ex vivo* treatment with Praziquantel

After collection, adult worm parasites were transferred to 24-well culture plates containing RPMI-1640 culture medium, 200 mM L-glutamine, 10 mM HEPES, 24 mM of $NaHCO_3$, 10,000 UI of Penicillin and 10 mg/mL of Streptomycin, from Sigma-Aldrich, pH 7, and supplemented with 15% fetal bovine serum. About five parasites, individually or as a couple, were added to each well and the same concentration of drug was used in two wells. All experiments were carried out in tree biological replicates, ten on each replicate (n = 30) for each studied group: 1) PZQ-susceptible male worms, 2) PZQ-susceptible female worms, 3) PZQ-resistant male worms, 4) PZQ-resistant female worms, 5) PZQ-susceptible couple worms, and 6) PZQ-resistant couple worms. Parasites were incubated overnight at 37 ºC in a 5% CO_2 atmosphere to recover from stress caused by perfusion. After this period, male and female worms were treated in culture with a dose of PZQ (0.3 μM, for 3 h) with impact in the parasite, but with the guarantee of not killing them, then washed twice with saline solution to clean any traces of culture medium and prepared for SEM. Couple worms of both strains were treated *ex vivo* with a lethal dose of PZQ (32 μM) for 48 h. During this period, worms were analyzed and monitored under an inverted optical microscope (DM-500, Leica), to evaluate and monitor the motility and viability of the worms, and morphological changes in eggs of the two parasite strains studied in this work. For negative control

of each group, worms were kept in RPMI-1640 drug free medium, under the same conditions.

3.4. Scanning electron microscopy

To evaluate tegumental morphologic changes in both strains of *S. mansoni* after *ex vivo* exposure to PZQ, adult worms were analyzed using SEM according to [30, 36, 37]. Briefly, worms were incubated at 37 ºC in a CO_2 atmosphere (5%) for 24 h. After incubation, the parasites were washed with 0.1 M of Sodium Cacodylate buffer pH 7.2 (for 1 h, changing the solution every 15 min), fixed in 2.5% Glutaraldehyde (pH 7.4) (Merck, Darmstadt, Germany) for 24 h and then fixed in 1% of Osmium Tetroxide for 1 h. Specimens were dehydrated in increasing concentrations of Ethanol (50%, 70%, 80%, 90%, 95% and 100%) for 30 min each, dried in a critical point dryer, mounted on stubs, metalized with gold particles using Sputter Coater and finally analyzed and photographed using an ultra-scanning electron microscope (Jeol-JSM-820).

3.5. Ethics statement

This research project was reviewed and approved by the Ethics Committee and Animal Welfare, Faculty of Veterinary Medicine, UL (Ref. 0421/2013). Animals were kept and handled in accordance with National and European legislation (DL 276/2001 and DL 314/2003; 2010/63/EU adopted on 22nd September 2010), with regard to animal protection and welfare, and all procedures were performed according to National and European legislation. The anesthetics and other techniques were used to reduce the pain and adverse effect of animal.

3.6. Statistical analysis

Results were expressed as mean ± SD. Data were statistically analyzed using the IBM SPSS Statistics software version 20.0 for Windows. Kolmogorov-Smirnov (Lilliefors significance correction) and Shapiro-Wilk tests were used to analyze data normality and Levene's test used to test homogeneity of variance. A t-test for independent samples was used to compare the average size of the eggs and lateral spines between susceptible and resistant strains, and Mann-Whitney (MW) test, to test whether there was difference between the ratios of lateral spines/eggs on the two strains studied. The level of significance was set at $p < 0.05$.

4. Results

4.1. *Ex vivo* effect of Praziquantel on *S. mansoni* PZQ-resistant and PZQ-susceptible strains

The viability of the adult worms was analyzed during *ex vivo* incubation with a concentration of 32 μM of PZQ for a 48 h period. We observed that the viability of adult worms resistant to PZQ and treated with this drug was similar to the negative resistant control group (resistant worms kept in RPMI-1640 drug free medium), in which all individuals were alive after the incubation period. We noticed that upon exposure to PZQ, adult worms from the resistant strain retracted, showed muscle contraction, reduction of movements (Figure III-2A-B) and, by the end of the incubation period, regaining motility (Figure III-2C), in comparison to the control group (Figure III-2D-F). Still, the worms remained alive and, after removal of the medium containing PZQ, they gradually regained full motility.

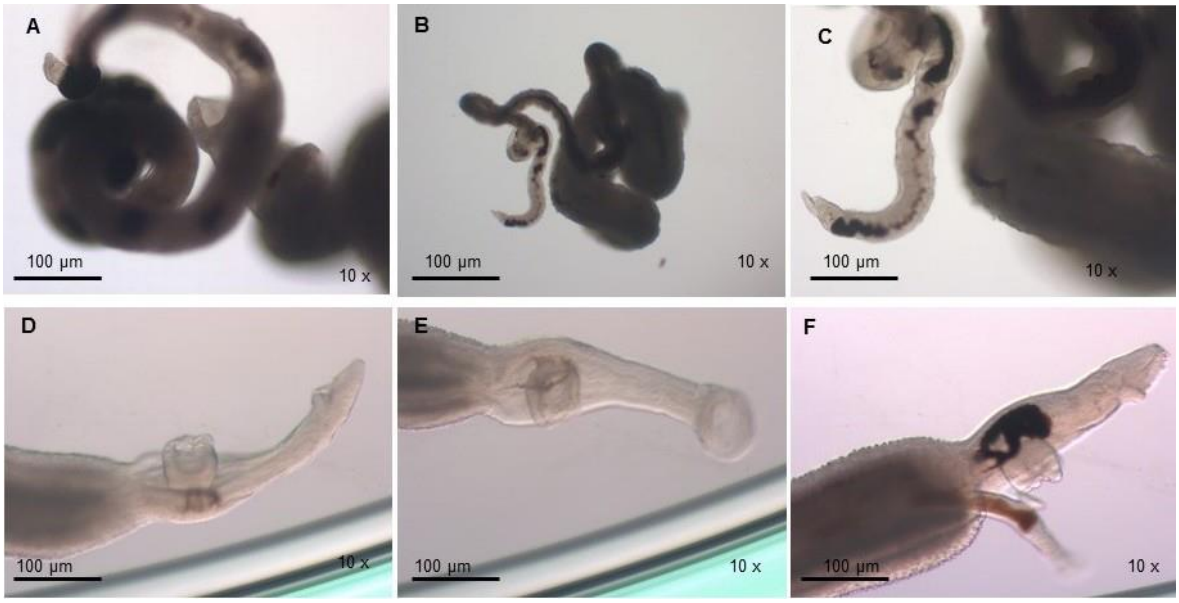

Figure III-2. Monitoring of *S. mansoni* resistant strain adult worms submitted to 32 μM of PZQ during 48 h. (A) Adult worms from the resistant strain exposed to PZQ, showing muscle contraction and reduction of movements, 6 h after PZQ-exposure; (B) Adult worms from the resistant strain exposed to PZQ, showing muscle contraction and little movements (24 h after drug exposure); (C) Adult worms from the resistant strain exposed to PZQ, began to gain some motility by the end of the incubation period (48 h); (D) Adult worms from the resistant strain not exposed to PZQ (negative control group - resistant worms kept in RPMI-1640 medium with no addition of the drug), 6 h of incubation period; (E) Adult worms from the resistant strain not exposed to PZQ (negative control group - resistant worms kept in RPMI-1640 medium with no addition of the drug), 24 h of incubation period; (F) Adult worms from the resistant strain not exposed to PZQ (negative control group - resistant worms kept in RPMI-1640 medium with no addition of the drug), at the end of the incubation period (48 h).

For susceptible parasites, where most of the worms were dead after drug addition, the viability was much lower compared to the negative susceptible control group (susceptible worms kept in RPMI-1640 drug free medium) and the treated-resistant parasite group. Susceptible strain parasites were more contracted (Figure III-3A) than those from the resistant isolate submitted to the same PZQ dose (Figure III-2A). After exposure to the drug, PZQ-susceptible adult worms retracted, showed muscle contraction, and reduction of movements (Figure III-3A), in comparison to the control group (Figure III-3C-D), and died after 24 h of incubation period (Figure III-3B). After removal of the medium containing PZQ, they did not recover motility, contrary to what happened with resistant strain parasites that gradually regained motility.

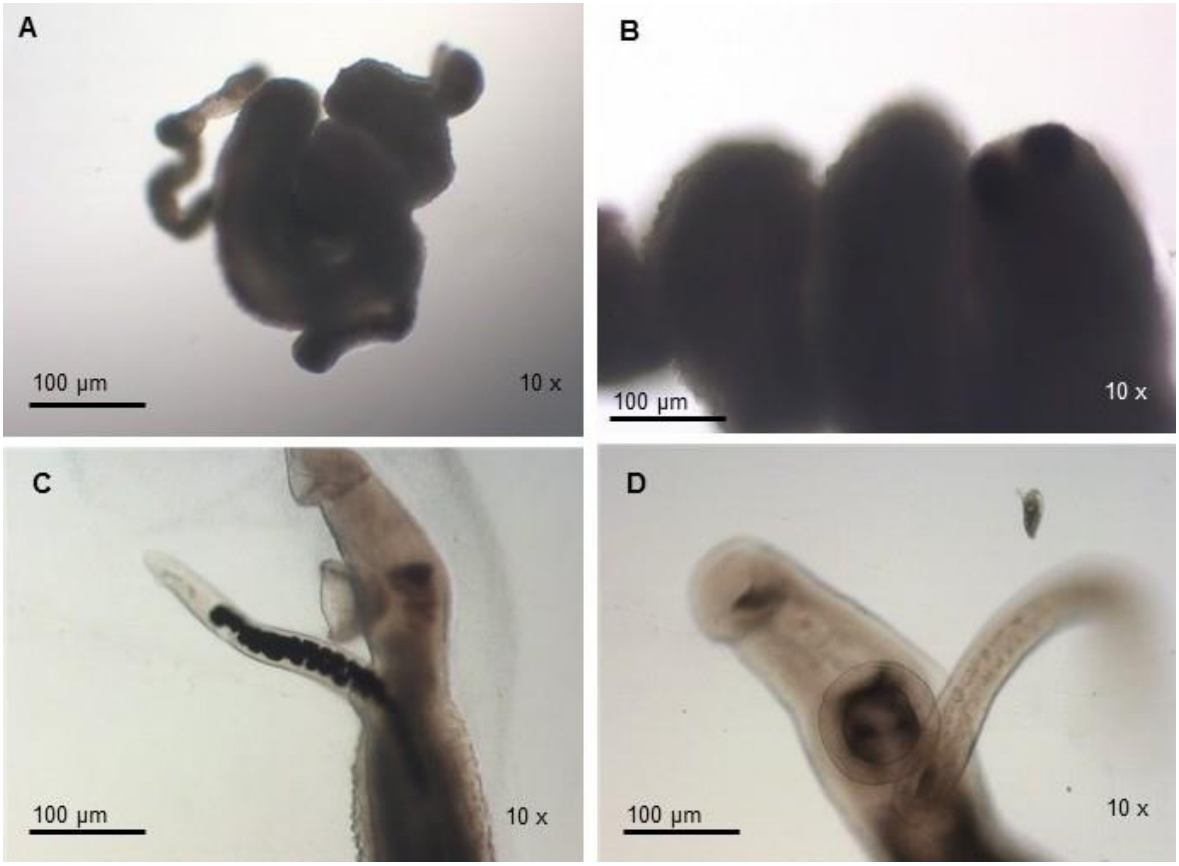

Figure III-3. Monitoring of *S. mansoni* susceptible strain adult worms submitted to 32 µM of PZQ during 48 h. (A) Adult worms from the susceptible strain exposed to PZQ, showing muscle contraction and reduction of movements, 6 h after PZQ-exposure; (B) Adult worms from the susceptible strain dead after exposed to PZQ, 24 h after exposure; (C) Adult worms from the susceptible strain not exposed to PZQ (negative control group - susceptible worms kept in RPMI-1640 medium with no addition of the drug), 6 h of incubation period; (D) Adult worms from the susceptible strain not exposed to PZQ (negative control group - susceptible worms kept in RPMI-1640 medium with no addition of the drug), 24 h of incubation period.

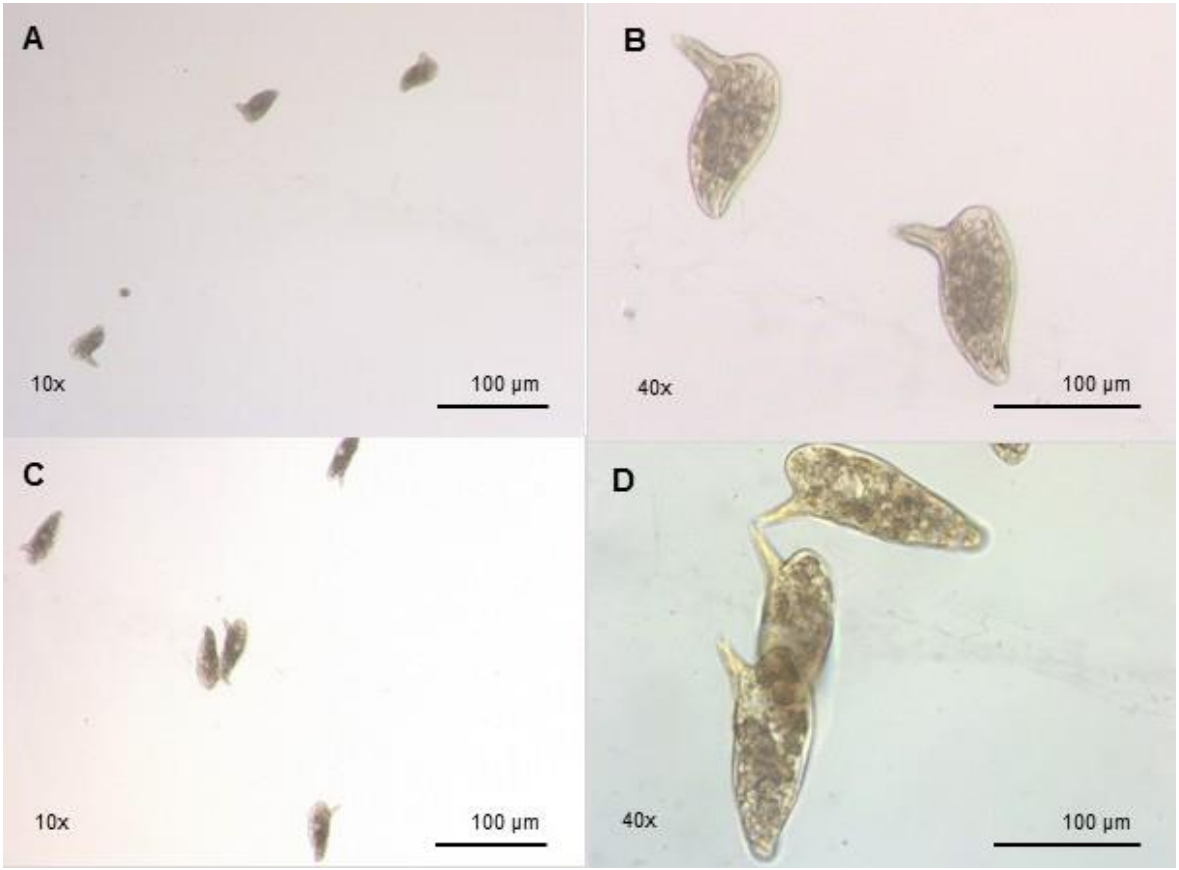

Figure III-4. Morphological difference between eggs from resistant strain and susceptible strain. (A) Eggs from resistant strain parasites, showing morphology alterations, smaller size and smaller lateral spines (10x); (B) Eggs from resistant strain, in a bigger scale, showing morphology alterations, smaller size, and smaller lateral spines (40x); (C) Eggs from susceptible strain parasites, showing normal morphology (10x); (D) Eggs from susceptible strain parasites, showing normal morphology, in a bigger scale (40x).

Regarding the egg morphology, it was different between PZQ-resistant and PZQ-susceptible stains (Figure III-4). Eggs from resistant parasite females (Figure III-4A-B, n = 7) were smaller ($p < 0.05$) when compared to those from the susceptible strain (Figure III-4C-D, n = 7; Figure III-5 and Table III-1). Furthermore, we found statistical significant differences in the size of the lateral spine of the eggs, those from resistant strain females had smaller ($p < 0.05$) and thicker lateral spines than those of susceptible worms (Figure III-5 and Table III-1). Moreover, the lateral spine of the eggs from resistant strain had the terminal portion less salient and acute, compared to the susceptible strain (Figure III-4). In order to confirm that the observed differences in lateral spine size were not a simple consequence of smaller size of the egg in resistant worms, the ratio between lateral spine and egg sizes was determined for resistant strain and susceptible strain (Table III-1). When the ratio of the two strains were compared, we found a significant difference ($p < 0.05$) between them, which means

that the difference observed in the size of the lateral spine is not only a consequence of smaller size of the eggs but perhaps of an alteration in their morphology. These interesting findings need to be explored, since they might have important repercussions on pathological and symptomatology effects induced by this strain.

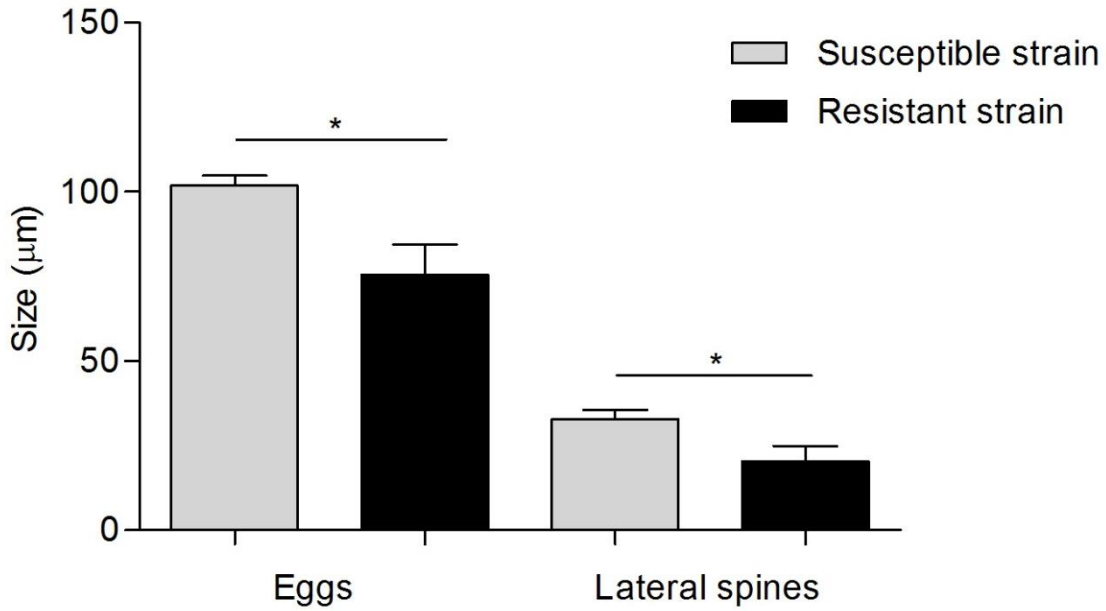

Figure III-5. Difference in egg morphology of *S. mansoni* resistant strain and susceptible strain. Gray bars – measurements of eggs and lateral spines from susceptible strain worms; Black bars – measurements of eggs and lateral spines from resistant strain worms. Data was presented as mean ± SD. Statistical analysis was performed by parametric t-test, for independent simples, whose level of significance was set at $p < 0.05$. *Indicates $p < 0.05$.

Table III-1. Difference in egg morphology (size of the eggs and lateral spines and ratio between them) of *S. mansoni* resistant and susceptible parasites (n = 7). SS – susceptible strain, RS – resistant strain.

Egg morphology	Size (µm) mean ± SD	95% Confidence Limits	*p*-value
Susceptible strain eggs	101.80 ± 2.99	99.03 – 104.57	p < 0.05 (t-test)
Resistant strain eggs	75.60 ± 8.98	67.29 – 83.91	
Susceptible strain lateral spines	32.78 ± 2.75	30.24 – 35.33	p < 0.05 (t-test)
Resistant strain lateral spines	20.26 ± 4.73	15.88 – 24.64	
Susceptible strain ratio (SS spine/SS egg)	0.32 ± 0.018	0.30 – 0.34	p < 0.05 (MW test)
Resistant strain ratio (RS spine/RS egg)	0.27 ± 0.036	0.23 – 0.29	

4.2. Effect of Praziquantel on tegument of *S. mansoni* PZQ-resistant and PZQ-susceptible strains

We analyzed the presence of tegumental alterations in *S. mansoni* PZQ-resistant strain adult worms and in the parental PZQ-susceptible strain upon addition of 0.3 µM PZQ during 3 h, using SEM. Significant changes were only observed in male and female worms of the susceptible strain. In susceptible males not exposed to PZQ, the oral and ventral sucker (Figure III-6A) and body surface (Figure III-6B-C) did not show any changes, while males exposed to the same drug presented changes in acetabular sucker (Figure III-7A), tegument peeling (Figure III-7B), and destruction of tubercles and spines (Figure III-7C-D).

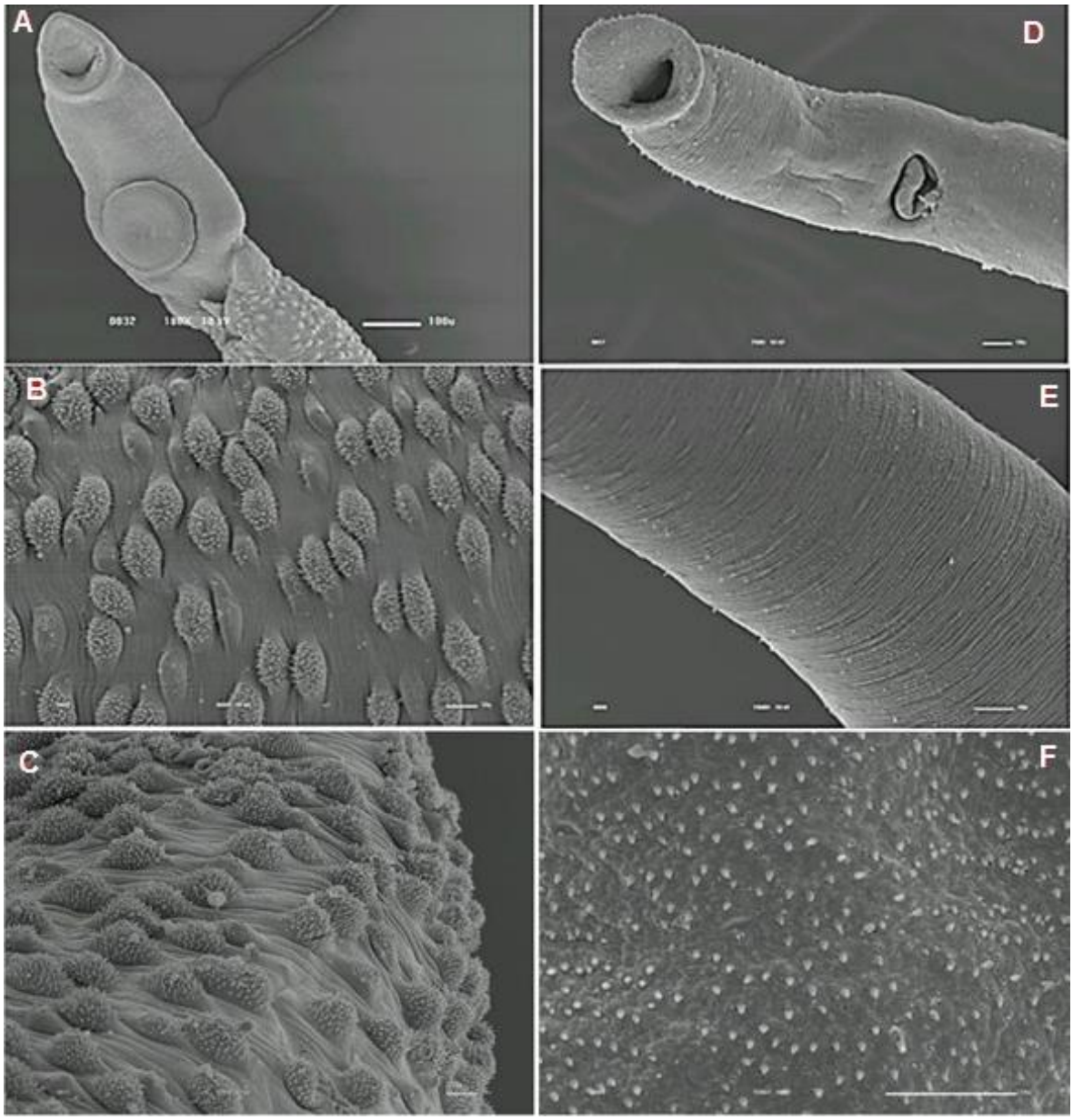

Figure III-6. Scanning electron microscopy of *S. mansoni* PZQ-susceptible strain. (A-C) Susceptible strain adult males of control group kept in RPMI-1640 drug free medium for 3 h, showing normal morphology of the tegument, and oral and ventral suckers; (D-F) Susceptible strain adult females of control group kept in RPMI-1640 drug free medium for 3 h, showing normal morphology of the tegument, and oral and ventral suckers. Image magnifications: (A) 180x 10 kV _____100 µm; (B) 900x 10 kV _____10 µm; (C) 800x 10 kV _____10 µm; (D) 500x 10 kV _____10 µm; (E) 1200x 10 kV _____10 µm; (F) 3300x 10 kV _____10 µm.

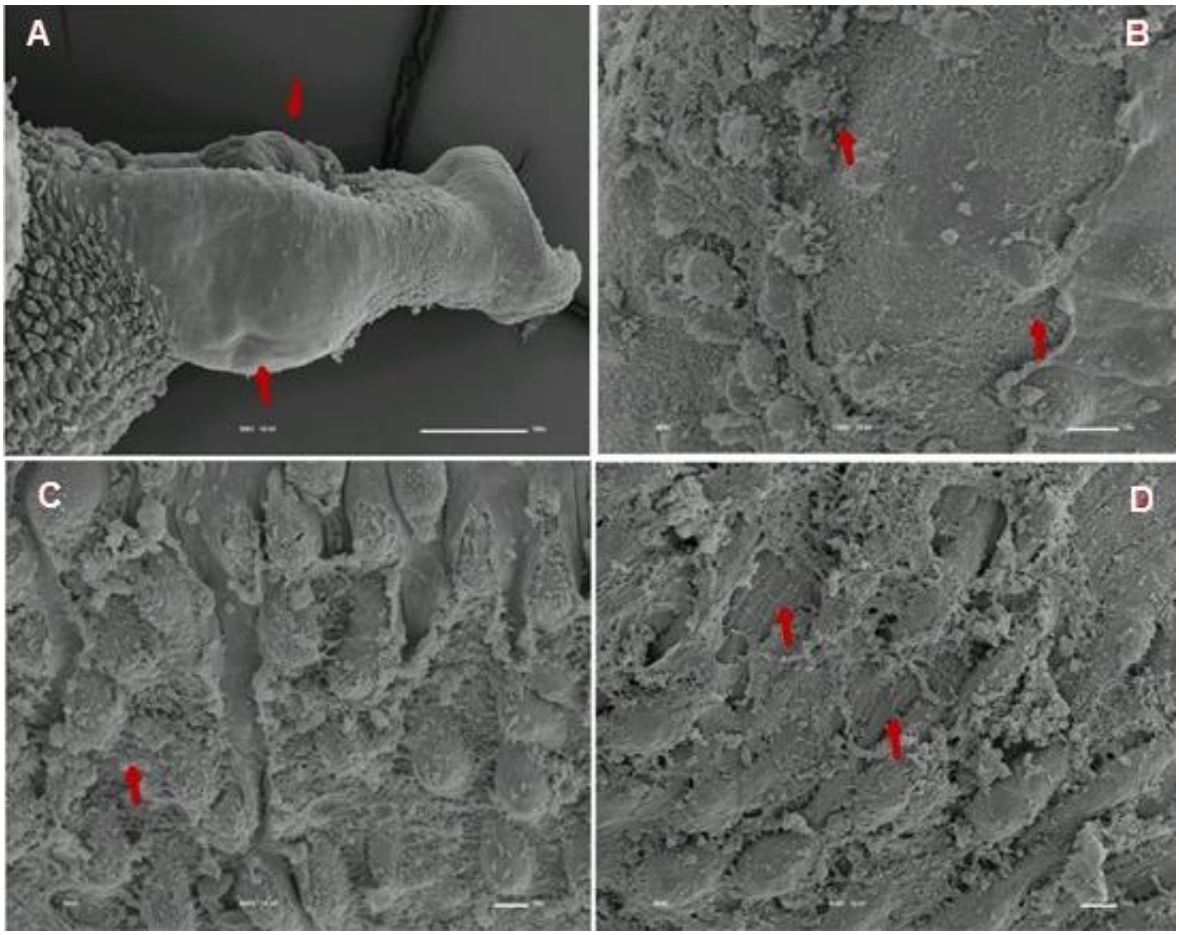

Figure III-7. Scanning electron microscopy of *S. mansoni* PZQ-susceptible strain adult males after exposure to 0.3 µM of PZQ for 3 h. (A) Susceptible strain adult males upon exposure to PZQ, presenting changes in acetabular suckers; (B) Tegument peeling; (C-D) Destruction of tubercles and spines. Red arrows indicate alterations. Image magnifications: (A) 300x 10 kV _____100 µm; (B) 1200x 10 kV _____10 µm; (C) 800x 10 kV _____10 µm; (D) 850x 10 kV _____10 µm.

Susceptible strain females not exposed to PZQ showed normal morphology of ventral and oral suckers (Figure III-6D) and normal morphology of the body surface (Figure III-6E-F), but when exposed to the drug, presented muscle contraction and corrugations (Figure III-8A-C). Furthermore, they showed alterations in the oral sucker (Figure III-8D) and peeling of some tegumental regions (Figure III-8E-F).

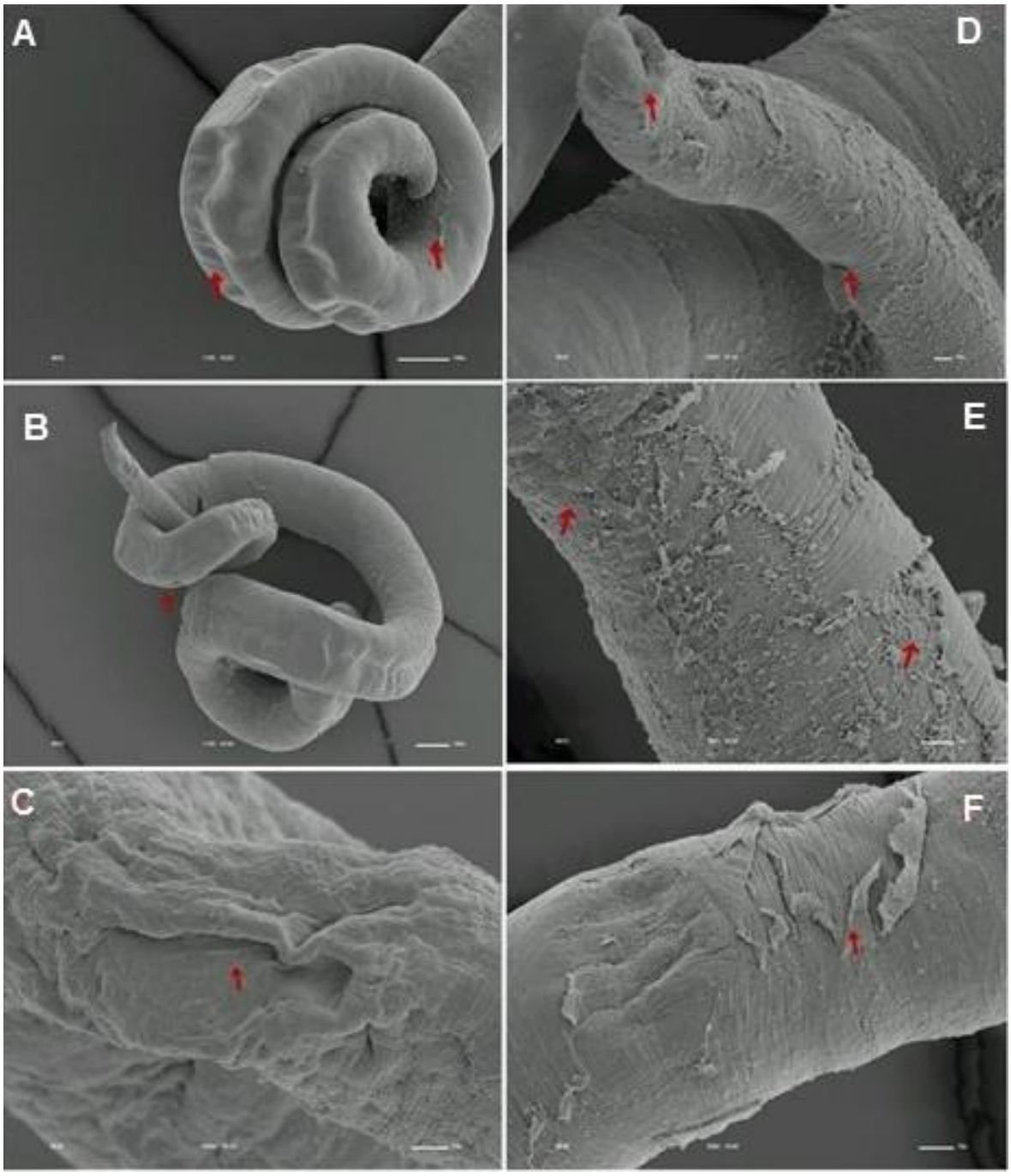

Figure III-8. Scanning electron microscopy of *S. mansoni* PZQ-susceptible strain adult females after exposure to 0.3 µM of PZQ for 3 h. (A-C) Susceptible strain adult females upon exposure to PZQ, showing muscle contraction and corrugations; (D) Alterations in oral sucker; (E-F) Peeling of some tegumental regions. Red arrows indicate alterations. Image magnifications: (A) 170x 10 kV _____100 µm; (B) 110x 10 kV _____100 µm; (C) 1000x 10 kV _____10 µm; (D) 500x 10 kV _____10 µm; (E) 900x 10 kV _____10 µm; (F) - 950x 10 kV _____10 µm.

Tegumental alterations induced by PZQ were not so significant in the resistant strain, when compared to the susceptible strain. As expected, the control group of resistant males did not show any tegumental alterations, showing normal morphology of the oral and ventral suckers (Figure III-9A-B) and tegument (Figure III-9C-F). When exposed

to PZQ, those worms presented small alterations in some areas, such as changes in oral and ventral suckers (Figure III-10A-C), and little alterations in the body surface, with losses of tubercles and spines (Figure III-10D-F).

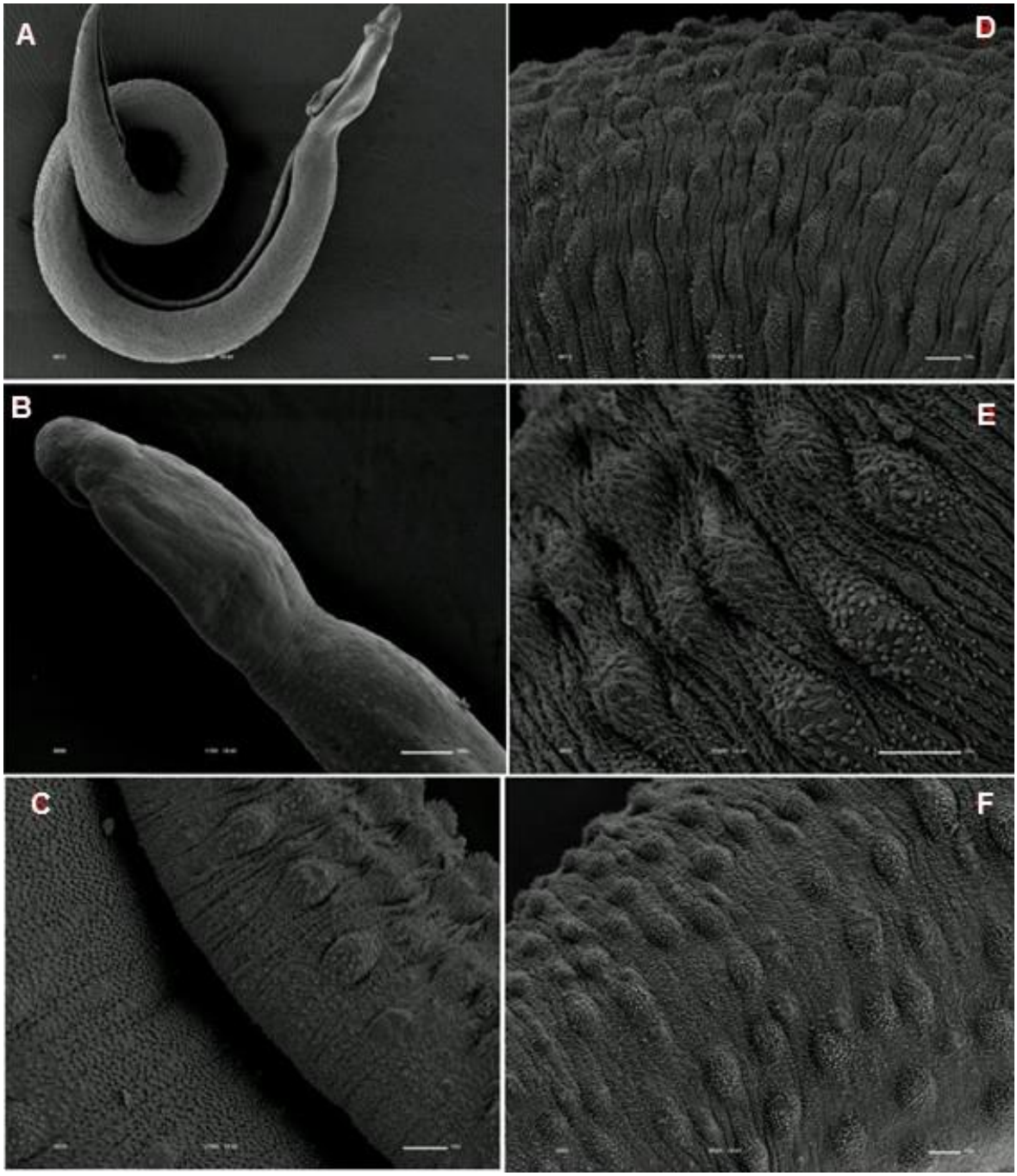

Figure III-9. Scanning electron microscopy of *S. mansoni* PZQ-resistant strain adult males of control group kept in RPMI-1640 drug free medium for 3 h. (A-B) Resistant strain adult males kept in RPMI-1640 drug free medium, showing normal morphology of the oral and ventral suckers; (C-F) Normal morphology of the tegument. Image magnifications: (A) 70x 10 kV _____100 µm; (B) 170x 10 kV _____100 µm; (C) 1200x 10 kV _____10 µm; (D) 950x 10 kV _____10 µm; (E) 2200x 10 kV _____10 µm; (F) 850x 10 kV _____10 µm.

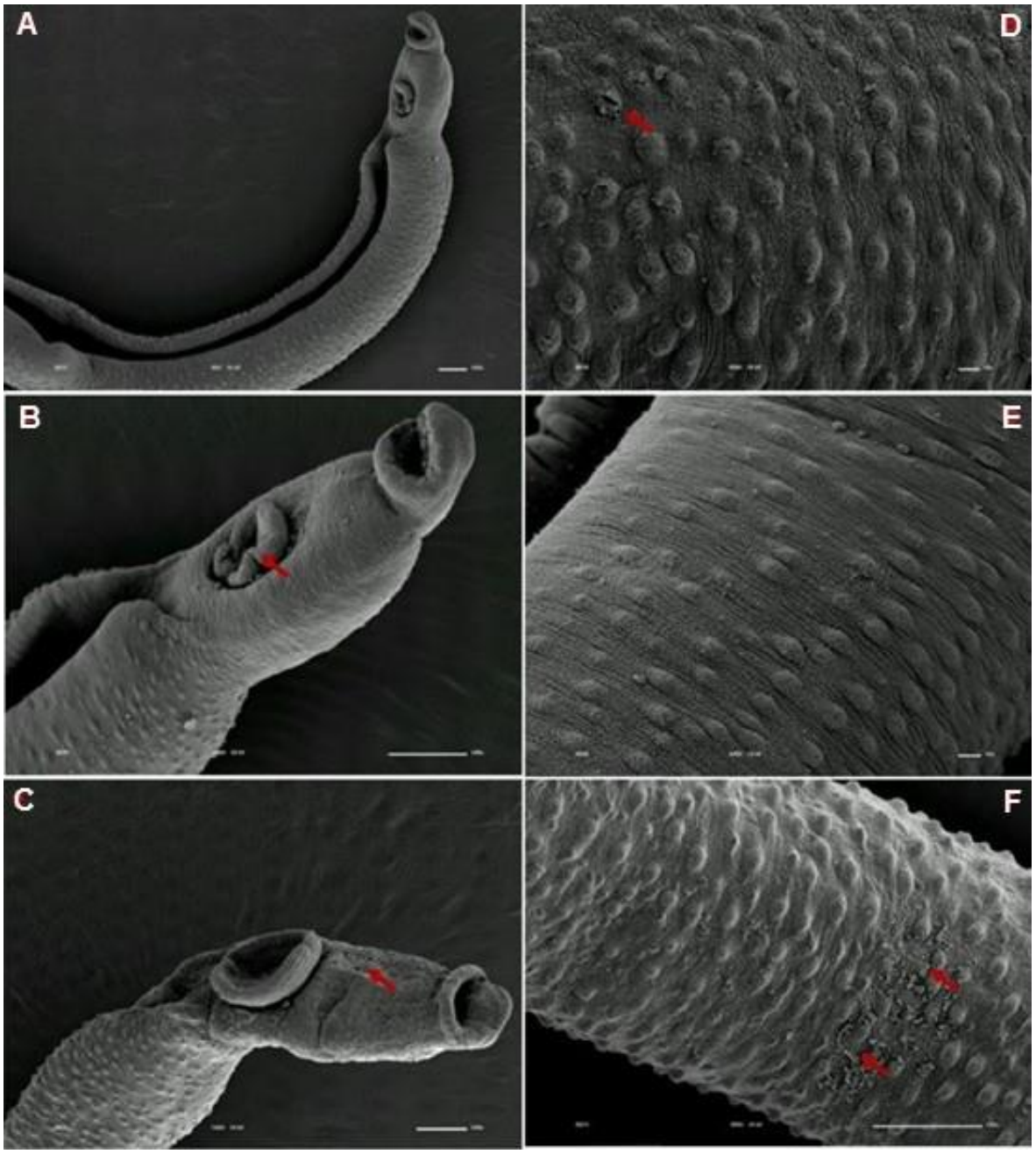

Figure III-10. Scanning electron microscopy of *S. mansoni* PZQ-resistant strain adult males after exposure to 0.3 μM of PZQ for 3 h. (A-C) Resistant strain adult males upon exposure to PZQ, presenting small changes in oral and ventral suckers; (D-F) Losses of tubercles and spines. Red arrows indicate alterations. Image magnifications: (A) 90x 10 kV _____100 μm; (B) 250x 10 kV _____100 μm; (C) 160x 10 kV _____100 μm; (D) 550x 10 kV _____10 μm; (E) 600x 10 kV _____10 μm; (F) 350x 10 kV _____100 μm.

Similarly female resistant worms control group did not present any tegumental damages, neither in oral and ventral suckers (Figure III-11A-B), nor in the tegument surface (Figure III-11C-D). Upon drug exposure, those worms only showed very few alterations, namely, some morphological changes in the oral sucker (Figure III-12A),

some light peeling in the worm ventral region (Figure III-12B), and alterations in some tegumental areas (Figure III-12C-D).

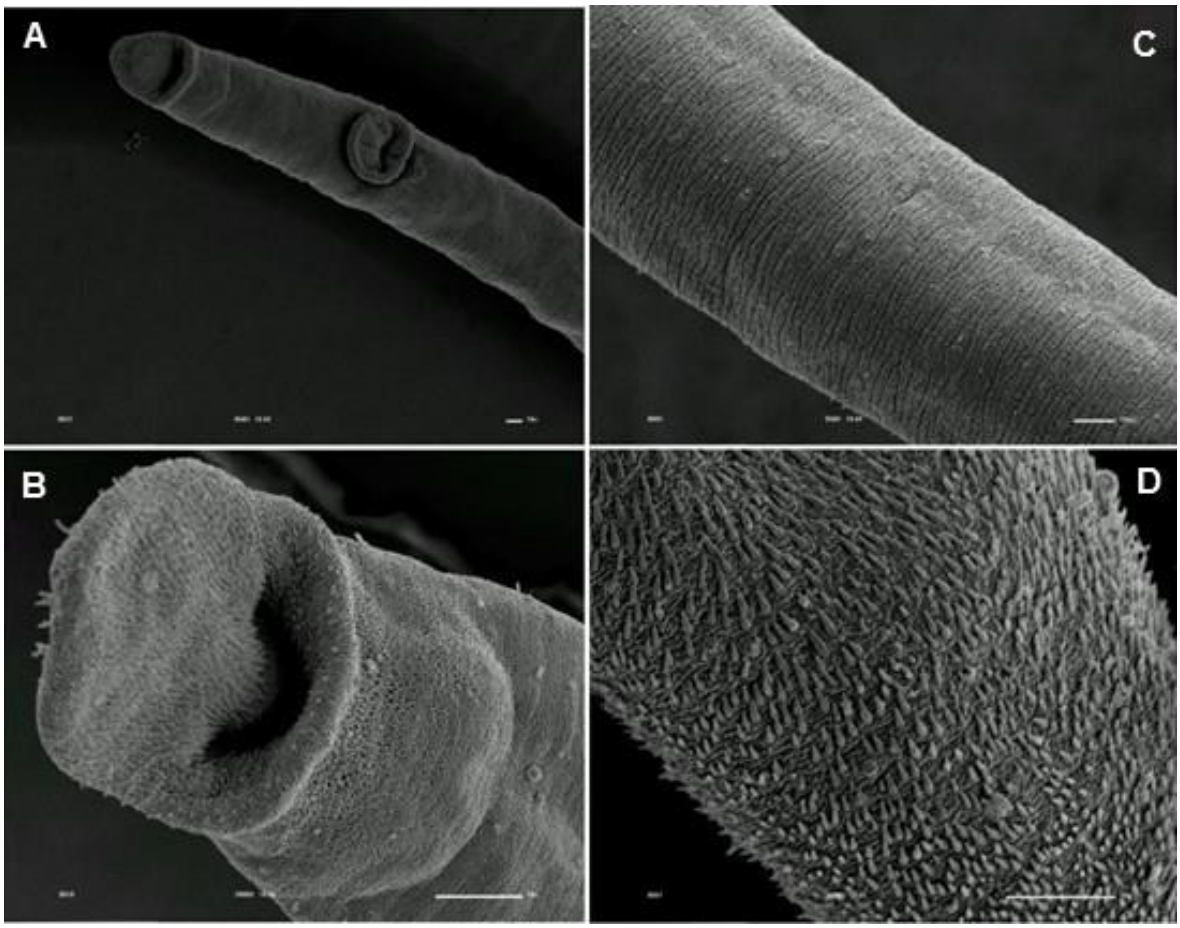

Figure III-11. Scanning electron microscopy of *S. mansoni* PZQ-resistant strain adult females of control group kept in RPMI-1640 drug free medium for 3 h. (A-B) Resistant strain adult females kept in RPMI-1640 drug free medium, showing normal morphology of the oral and ventral suckers; (C-D) Normal morphology of the tegument. Image magnifications: (A) 450x 10 kV _____10 µm; (B) 2000x 10 kV _____10 µm; (C) 950x 10 kV _____10 µm; (D) 2500x 10 kV _____10 µm.

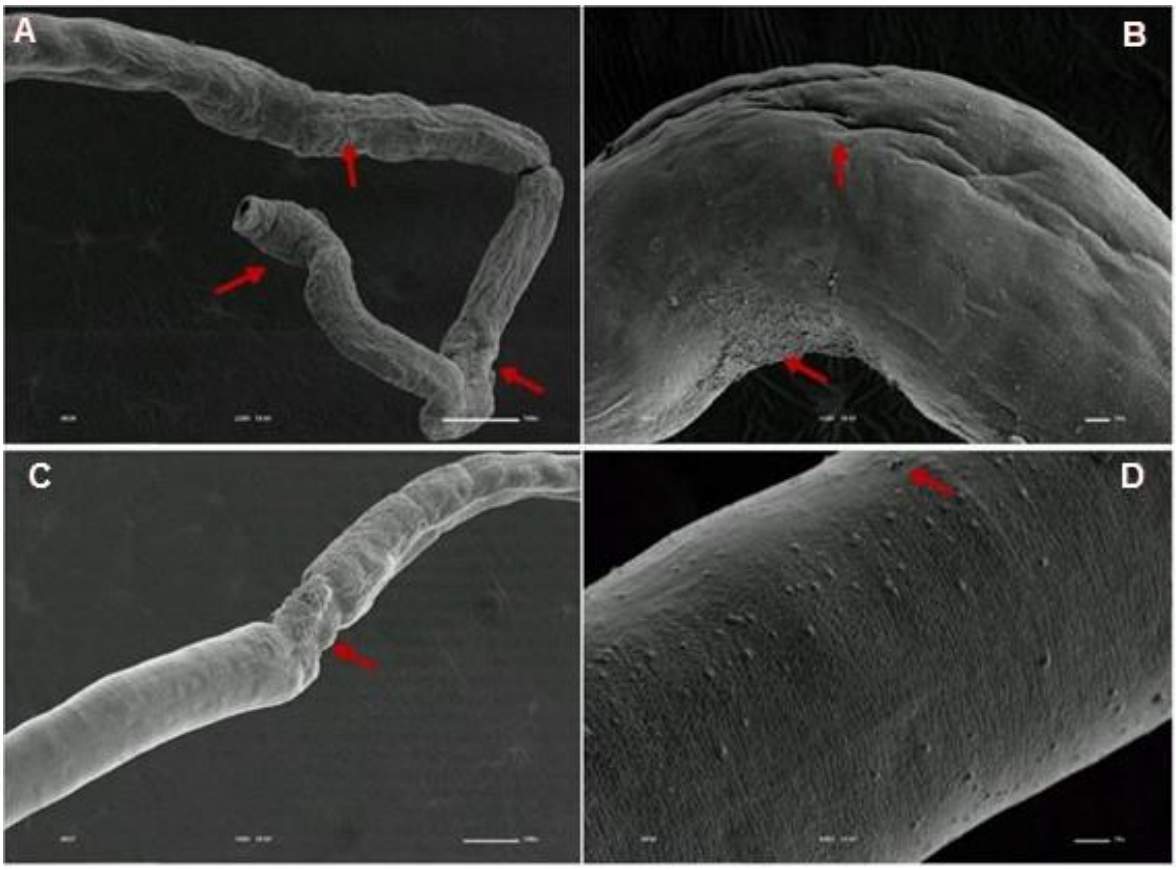

Figure III-12. Scanning electron microscopy of *S. mansoni* PZQ-resistant strain adult females after exposure to 0.3 µM of PZQ for 3 h. (A) Resistant strain adult females upon exposure to PZQ, presenting changes in oral suckers; (B) Light peeling in the worm ventral region; (C-D) Alterations in some tegumental areas. Red arrows indicate alterations. Image magnifications: (A) 220x 10 kV _____100 µm; (B) 550x 10 kV _____10 µm; (C) 160x 10 kV _____100 µm; (D) 900x 10 kV _____10 µm.

5. Discussion

In past years, several studies have been performed in order to demonstrate that resistance/tolerance to PZQ may occur and is more than hypothetical [26-29]. Our group in particular had selected, by stepwise drug pressure, a *S. mansoni* strain that is isogenic to its parental fully susceptible counterpart, except for genetic determinants accounting for the PZQ-drug resistance phenotype, and phenotypically similar to the susceptible strain except in resistance [32]. In the present study, we took the advantage of the availability of these two strains of *S. mansoni* and performed a comparative assessment of morphological alterations that the *ex vivo* effect of PZQ can cause on *S. mansoni* PZQ-resistant parasites. Some authors [38, 39] have already performed studies with PZQ-resistant isolates obtained from an Egyptian and a Senegalese patient eggs, which were not cured by three therapeutic doses of PZQ, where they demonstrated that isolates from resistant infections were less susceptible to PZQ-induced tegumental damage *ex vivo* [38] and PZQ-resistant isolates may be more pathogenic in mice than the susceptible ones [39]. However, as far as we know, this is the first report in which the *ex vivo* effect of PZQ on the morphological characteristics of a resistant strain of *S. mansoni* can be compared with its parental susceptible strain.

Parameters, such as motor activity, eggs morphology and tegumental changes, are often evaluated as indicators of biological activity in studies using schistosome species [38, 40-48], hence we evaluate these parameters in order to assess the effect of PZQ on adult *S. mansoni* PZQ-resistant and PZQ-susceptible worms' survival and fitness.

Our study shows that resistant strain worms have less muscle contraction and movements after exposure to PZQ than susceptible isolates. This complies with studies suggesting that contraction of somatic musculature is a marked effect of addition of PZQ to schistosomes *ex vivo* [38], and that worms resistant to PZQ *in vivo* have significantly reduced contractile responses to PZQ *ex vivo* [27]. It is also evident that, after removal of the medium containing the drug, resistant worms recover motility, unlike susceptible worms, where the majority of which are dead.

The tegument is a very important organ for schistosomes, for many reasons: it is important for the survival of the worms in the host [49-51], protecting the parasite against the action of the host's immune system, absorbing nutrients and molecules

and participating in synthesis of some proteins [52-54]. Some studies carried out many years ago, tried to clarify the action mechanisms of drugs used in schistosomiasis treatment. It has been shown that, worms subjected to PZQ, have vacuolization of the tegument and disruption of the apical tegumental layer [55]. Female worms present tegumental damage, such as, tegument and sub-tegument vacuolization and tegument and musculature destruction, while male worms show more pronounced and extensive surface alterations, which include surface bleeding, swellings, wrinkling, constrictions and surface lesions, particularly on the spined tubercles [56].

As shown by William and colleagues [38], our findings demonstrate that tegumental damage caused by the *ex vivo* effect of PZQ is much less evident in resistant strain adult worms than in the susceptible strain. Contrarily to what occurs in resistant parasites, in which PZQ does not seem to cause major damage, males of the susceptible strain present tegument peeling, tubercle and spine destruction and vesicles around the tubercles while females display peeling and wrinkling of the tegument and destruction of oral and acetabular suckers. These suggest that there might be a difference in the resistant strain tegument composition that may render the worms less responsiveness to PZQ. Therefore, it will be very interesting to perform a more in-depth study of the resistant strain tegument, using for instance the transmission electron microscopy in order to analyze the ultrastructure of the resistant strain tegument, allowing to see if PZQ resistance might in any way influence pathology symptomology, since one of the hallmark effects of PZQ on schistosomes *ex vivo* is the disruption of surface tegument [38]. It is important to notice that susceptible strain females presented much less damage than males. This observation is in agreement to the findings described in the previous Chapter where females appeared more tolerant to PZQ [32]. In the resistant strain, damages on both males and females were so small that it is far more difficult to make a similar comparison.

It has been stated in literature that the oviposition of *S. mansoni* during *in vitro* culture of adult worm show three very distinct phases in the kinetics of oviposition: an initial phase with low egg production, a period of maximum oviposition and finally a gradual reduction in the number of eggs during the last phases of culture [57]. Liang and colleagues [39] demonstrated that mice infected with PZQ-resistant isolates shed more eggs in their feces than those carrying drug-susceptible parasites and mice infected with any of the resistant isolates also had larger numbers of eggs in their tissues. Mice

infected with our PZQ-resistant strain shed more eggs than those infected by the counterpart susceptible strain [Personal observation]. This is important because if there is a change in the biological characteristics of schistosomes associated with the development of resistance to PZQ, it could affect the transmission and pathology of the disease they cause [39].

Another interesting finding observed in this study is that resistant strain eggs have different morphology, with smaller size and a smaller lateral spine (less salient and acute) in comparison with eggs from susceptible strain, which may have repercussions in the pathology of the disease, once the eggs are the major cause of pathology in schistosomiasis [58]. We have observed that some mice infected with the PZQ-resistant strain used here presented some neurological manifestations including deviation of the head, tendency to roll over on stimulation, ataxia, and convulsions, very similar to what is usually seen in mice affected with cerebral malaria [59]. Based on this, it is not unreasonable to speculate that resistant strain parasites might have altered tissue tropism, namely, a higher tropism for brain or spinal cord, which may potentiate the development of neurological manifestations. Clearly, further studies on mice infected with PZQ-resistant isolates are required to confirm this hypothesis.

In conclusion, we compared morphological characteristics of *S. mansoni* PZQ-resistant and PZQ-susceptible strains upon addition of this drug *ex vivo*. It was demonstrated that the resistant strain presents i) less muscular contractions, ii) less tegumental damage, iii) more viability, and iv) recovering motility when the drug is removed, indicating fully active life after a PZQ treatment is ceased. The resistant strain demonstrated different egg morphology when compared with susceptible strain. Those are important findings since any biological changes can produce relevant alterations in the transmission and pathology of diseases. Comparing two strains that only differ in resistance characteristics is an important step in the study of schistosomiasis as it guarantees that the differences observed between the two strains are closely related to resistance. Increase tolerance/resistance to PZQ in *in vivo* experiments is an obvious fact and studies should be performed to clarify the mechanisms associated with it. This study certainly opens doors for further in-depth *S. mansoni* drug resistance studies.

6. References

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CHAPTER IV – RESEARCH WORK 3

IV. Comparative proteomics on Praziquantel-resistance in *S. mansoni*

Adapted from: Pinto-Almeida, A., Mendes, T., Ferreira, P., Belo, S., Anibal, F.F., Allegretti, S.M., Carrilho, E., and Afonso, A. Comparative proteomics reveals charcteristic proteins on Praziquantel-resistance in *Schistosoma mansoni*. *Submitted manuscript.*

1. Abstract

The extensive use of PZQ, the only drug available to treat schistosomiasis, has brought concern about the emergence of PZQ-resistance/tolerance by *Schistosoma* spp., thus reaffirming an urge for the development of new treatment alternatives. Studies of *Schistosoma* spp. genome, transcriptome and proteome are crucial to better understand this situation. By stepwise drug pressure from a fully susceptible parasite strain, our group selected a *S. mansoni* variant strain stably resistant to PZQ and isogenic to its fully susceptible parental counterpart, except for the genetic determinants of PZQ-resistance phenotype. Based on this, the objective of this study was to compare the proteomes of both strains, identifying proteins from male and female adult worms of PZQ-resistant and PZQ-susceptible strains, exposed and not exposed to PZQ, which were separated by high-resolution two-dimensional electrophoresis and sequenced by high throughput LC-MS/MS. This study identified 60 *S. mansoni* proteins, some of which differentially expressed in either strain. This information represents substantial progress towards deciphering the worm proteome. Furthermore, these data may constitute an informative source for further investigations into PZQ-resistance and increase the possibility of identifying proteins related to this condition, possibly contributing to avoid or decrease the likelihood of development and spread of PZQ-resistance.

2. Introduction

Schistosomiasis is one of the most important infectious parasitic diseases mainly in sub-Saharan Africa [1, 2]. Despite many efforts to control its transmission [3-5], essentially after the introduction of a chemotherapeutic treatment in 1980s, the disease is still highly prevalent [6]. Nowadays, this control is based on PZQ the only drug available for chemotherapy [7]. Treatment with PZQ is effective and inexpensive [7], but frequent schistosome reinfection occurs in endemic areas and may cause irreversible damages to the liver, kidneys, or urinary tract [8]. Because of its high prevalence, schistosomiasis has earned a Category II disease, ranking next to malaria, for importance as a target tropical disease by the World Health Organization Special Program for Research and Training in Tropical Diseases [8].

Although the impact of schistosomiasis could be dramatically reduced by improvement in education and sanitation for humans and elimination of the intermediate host snails, such methods are not sufficient to control or eradicate this parasitosis. In the absence of vaccines, the control of this disease relies on chemotherapy to ease symptoms and reduce transmission. The increasing reliance on mass PZQ administration programs has exerted selective pressure on parasite population and PZQ-resistance/loss of susceptibility is being described by different investigators [9-11]. With no alternative drugs or vaccines, the fight against schistosomiasis could become a huge battle [12].

Identification of proteins is very important for understanding how schistosomes regulate host immune systems to establish chronic infections and also elucidate other aspects of parasite-host interaction [8]. Furthermore, a comprehensive deciphering of the schistosome genome, transcriptome, and proteome has become increasingly central for understanding the complex parasite-host interplay [13, 14]. Therefore, such information can be expected to facilitate the discovery of vaccines and new therapeutic drug targets, as well as new diagnostic reagents for schistosomiasis control [8, 13, 14], and may aid the development of protein probes for selective and sensitive diagnosis of schistosomiasis [15].

Proteomics approaches encompass the most efficient and powerful tools for identification of protein complexes [16-18] and have been widely used to decipher the proteome of parasites such as nematodes [19] and trematodes [20-27]. For *Schistosoma* spp., the proteome has been studied in many developmental life stages,

including schistosomula [28, 29], cercariae [29-31], egg [29, 32] and adult worm [29, 33-36]. But to our knowledge, *S. mansoni* PZQ-resistant strain proteome has not been yet reported and a schistosomiasis mansoni coherent screening for proteins related to PZQ-resistance is still necessary. Understanding the development of PZQ-resistance in *S. mansoni* is crucial to prolong the efficacy of the current drug and develop markers for monitoring drug resistance. It would also be beneficial in the design of new chemotherapeutic agents to overcome or prevent resistance, and in the identification of new drug targets.

As described in the Chapter II, our research group recently developed a resistant strain of *S. mansoni* that tolerates up to 1,200 mg PZQ/kg of mouse body weight. This *S. mansoni* variant strain was selected from a fully susceptible parasite strain, by stepwise drug pressure, and is isogenic, except for the genetic determinants of PZQ-resistance phenotypes, and significantly different of the counterpart *S. mansoni* susceptible strain [37]. As such, this *S. mansoni* PZQ-resistant strain represents a distinct and valuable model for the study of PZQ-resistance.

The present study intended to analyze, for the first time, the proteome of *S. mansoni* PZQ-resistant adult worms and compare it with its parental fully susceptible strain, using a high throughput LC-MS/MS identification. Therefore, this study could possibly represent a substantial progress toward deciphering the worm proteome, and may constitute an informative source for further investigations into PZQ-resistance, increasing the possibility of avoid or decrease the likelihood of development and spread of PZQ-resistance.

3. Material and Methods

3.1. Parasite samples

Two different parasite isolates were used in this study, the *S. mansoni* BH PZQ-susceptible strain (SS) and a stable PZQ-resistant strain (RS) obtained from the same BH strain as described in Chapter II [37]. These two parasite strains are routinely kept in their intermediate host *B. glabrata* snails at IHMT/UNL.

Mus musculus CD1 line male mice was chosen as the animal model for *S. mansoni* infection, because it is a good host for this parasite mimicking the *S. mansoni* human infection [38]. Mice infection occurred by natural transdermal penetration of cercariae, by exposing mice tails to about 100 cercariae of *S. mansoni* each.

Eight to ten-weeks adult worms were recovered by perfusion of the hepatic portal system and mesenteric veins, according to [39], and washed twice in RPMI-1640 medium (Sigma-Aldrich), to remove contaminating hair and blood clots.

It was analyzed males and females in separate, not exposed and exposed to PZQ, for RS and SS. Regarding to the groups of adult worms exposed to PZQ (EPZQ), after collecting, the parasites were transferred to 24-well culture plates containing RPMI-1640 culture medium, 200 mM L-glutamine, 10 mM HEPES, 24 mM of $NaHCO_3$, 10,000 UI of Penicillin and 10 mg/mL of Streptomycin, from Sigma-Aldrich, pH 7 and supplemented with 15% fetal bovine serum. Adult worms of the parasite were added on each well for each studied group for PZQ treatment: 1) PZQ-susceptible males (SM); 2) PZQ-susceptible females (SF); 3) PZQ-resistant males (RM), and 4) PZQ-resistant females (RF). Adult parasites were treated in culture, with 0.3 μM of PZQ during 24 h and then washed twice with saline solution to clean any traces of culture medium and stored in Trizol (Invitrogen, Carlsbad, California, USA) at -80 ºC, for posterior protein extraction. For the groups of adult worms not exposed to PZQ (NEPZQ), worms were kept in RPMI-1640 medium with no addition of drug, then washed twice with saline solution to clean any traces of culture medium and also stored in Trizol (Invitrogen, Carlsbad, California, USA) at -80 ºC, for posterior protein extraction. Accordingly, the experimental set up consisted of eight sample groups, four for parasites not exposed to PZQ (RM-NEPZQ, RF-NEPZQ, SM-NEPZQ and SF-

NEPZQ) and four for parasites exposed to PZQ (RM-EPZQ, RF-EPZQ, SM-EPZQ and SF-EPZQ).

3.2. Preparation of protein extracts

Schistosoma mansoni adult worm protein extracts were obtained using Trizol (Invitrogen, Carlsbad, California, USA) protocol, according to the manufacturer's instructions. Briefly, the parasites were lysed and homogenized directly in Trizol reagent at room temperature. The homogenized samples were incubated at room temperature to permit complete dissociation of the nucleoprotein complex. After homogenization, we proceeded to separation phase, adding Chloroform and centrifugation of samples. The aqueous phase was removed and the interphase and organic phenol-chloroform phase was used for protein isolation procedure. Next, Isopropanol precipitation was performed and the pellet was solubilized in SBI buffer [7 M Urea, 2 M Thiourea, 15 mM 1,2-diheptanoyl-snglycero-3-phosphatidylcholine, 0.5% Triton X-100, 20 mM Dithiotheitol (DTT) and Complete Mini Protease Inhibitor Cocktail Tablets], according to [40], and stored at -80 ºC until use.

Protein concentration in protein extracts was measured by Bradford assay [41] and the quality of the extract was verified in 12% uniform SDS-Polyacrylamide gel electrophoresis (PAGE).

3.3. Two-dimensional electrophoresis

Each experiment with two-dimensional electrophoresis (2-DE) gels was performed in triplicate with 240 µg of proteins, for each group. To prepare samples for 2-DE, protein samples in the mentioned concentration were diluted in rehydration solution containing 7 M Urea, 2 M Thiourea, 4% CHAPS (3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate), 0.5% IPG buffer, 1% DTT (Sigma), and 0.002% Bromophenol blue (Sigma). The rehydration was carried out passively overnight during 12 h in a 13 cm, pH 3-10 strip (Immobiline Drystrips, GE Healthcare). The strips were then applied on an Ettan IPGphor 3 (GE Healthcare) system, for protein separation by isoelectric focusing (IEF) following a typical IEF protocol, which involved three focusing steps at a constant 50 µA/strip: 3 h gradient to 3,500 V, 3 h at 3,500 V and finally 64,000 V h, until the end.

After focusing, the strips containing protein were reduced in an equilibration solution (50 M Tris HCl, pH 8.8, 6 M Urea, 20% Glycerol, 2% SDS) containing 2% DTT, and then alkylated in the same solution containing 2.5% Iodoacetamide (Sigma). The Immobilized pH gradient (IPG) strips and molecular weight standards were then transferred to the top of 12% uniform SDS-PAGE gels and sealed with 0.5% agarose. The second dimension was carried out using a Protein Plus Dodeca cell system (Bio-Rad) under an initial current of 15 mA/gel for 15 min, followed by increasing the current to 50 mA/gel until the end of the run (the dye front reached the bottom of the gel).

For 2-DE experiments, at least three replica of two dimensional polyacrylamide gel electrophoresis were performed for each group, confirming the reproducibility of the experimental procedure. Gels were fixed in 40% Methanol/10% Acetic Acid solution and stained with Coomassie Brilliant Blue R-350 (GE Healthcare). The spots were normalized and evaluated by the software ImageMaster 2D Platinum 7.0 (GE Healthcare).

3.4. In-gel digestion and peptide preparation for mass spectrometry analysis

The selected protein spots from the three replicate SDS-PAGE gels of each group were manually excised, distained, reduced, alkylated and digested in gel with Trypsin (Promega, Fitchburg, Wisconsin, USA). First, spots were washed in Milli-Q water, and then distained in a distaining solution containing 50% Methanol/2.5% Acetic Acid in purified water for 2 h at room temperature. This step was repeated until clear of blue stain. The gel fragments were incubated in 100% Acetonitrile (ACN) with occasional vortexing, until gel pieces became white and shrank. Then, the solution was removed and spots were completely dried, and ready for digestion. The in-gel digestion with Trypsin-modified sequencing-grade reagents (Promega) was done according to [42]. Briefly, protein digestion was conducted at 37 ºC overnight. After the incubation, the supernatant was transferred to a clean tube and 30 µL of 5% Formic Acid (FA)/60% ACN were added to gel spots for the extraction of tryptic peptides. This procedure was performed 2 x 30 min under constant agitation. The supernatant was pooled to the respective tube containing the initial peptide solution. This solution was dried in a SpeedVac (Thermo Scientific) and the peptides were re-suspended in 8 µL of 0.1% FA. The peptides were desalted in reverse phase micro-columns Zip-Tip C18

(Millipore), according to manufacturer's instructions. Peptides were dried again and re-suspended in 50% ACN/ 0.1% Trifluoracetic Acid (TFA) solution.

3.5. Peptide analysis by LC-MS/MS and protein identification

The digested peptides were analyzed by LC-MS/MS using a nano-LC system (EASY-nLC II, Thermo Scientific), coupled online to a hybrid mass spectrometer ion trap linear-Orbitrap (LTQ Orbitrap Velos, Thermo Scientific) using an ion nanospray source, namely, Nano-Flex II nanospray (Thermo Scientific). The samples were injected (10 µL/min, 4 min) in a pre-column (C18, 100 µm DI x 2 cm, Thermo Scientific), and then eluted under flow of 300 nL/min using an elution gradient to a C18 column (10 cm × 75 µm DI, 3 µm, 120 Å, Thermo Scientific). All LC-MS/MS data (in RAW format) were acquired using XCalibur software, version 2.0.7 (Thermo Fisher Scientific) and converted in .mgf files using MassMatrix MS Data File Conversion version 3.9. The analyzes were performed in scan mode in the range of 400-2000 m/z; positive mode; capillary voltage of 4500 V; nebulizer to 8.0 psi; drying gas at a flow of 5.0 L/min and at 220 ºC of evaporation temperature of the spray.

The list of peptide and fragment mass values generated by the mass spectrometer for each spot were submitted to a MS/MS ion search using the Mascot 2.0 online search engine (Matrix-Science) to search the LC-MS/MS data against the NCBInr database *Schistosoma_mansoni*_NCBI_112014, November 2014. The database was downloaded and transferred to a searchable database for MS/MS data on an in-house Mascot server. The parameters used were: allowance of two tryptic miss cleavages, peptide mass tolerance of ±0.6 Da, fragment mass tolerance of ±0.2 Da, peptide charge +1, variable modifications of methionine (oxidation), and fixed modifications of cysteine (carbamidomethylation). To avoid random matches, only ions with individual score above of the indicated by the Mascot to identity or extensive homology ($p < 0.05$) were considered for protein identification. However, when the Mascot score was not significant, but the percentage coverage and root mean squared error (RMSE) were in the same range as those of proteins with a significant match, proteins were deemed identified if additional parameters, such as its calculated p*I* and *M*w, were in agreement with those observed for the actual gel spot and the species matched was *S. mansoni*, according to [29].

The molecular function and biological process were assigned for the proteins identified according to information obtained from the Gene Ontology (GO) database [43, 44]. The exact annotation for each protein was used in most cases. However, the catalytic activity category was used for all proteins with molecular function associated with (GTPase, hydrolase, isomerase, kinase, ligase, lyase, oxidoreductase, transcription and transferase activities). Binding category was used for all types of ligand identified (actin, ATP, Calcium, GTP, magnesium ion, metal ion, protein domain specific and nucleotide bindings). Furthermore, there was other molecular function categories classified such as chaperone, motor, regulation of muscle contraction, structural and transport. The proteins that had no associated known function were classified as “unknown”.

3.6. Ethics statement

This research project was reviewed and approved by the Ethics Committee and Animal Welfare, Faculty of Veterinary Medicine, UL (Ref. 0421/2013). Animals were maintained and handled in accordance with National and European legislation (DL 276/2001 and DL 314/2003; 2010/63/EU adopted on 22nd September 2010), with regard to the protection and animal welfare, and all procedures were performed according to National and European legislation. The anesthetics and other techniques were used to reduce the pain and adverse effect of animal.

4. Results

4.1. 2-DE separation of proteins from *S. mansoni* PZQ-resistant and PZQ-susceptible adult worms

As said in the Material and Methods section of this Chapter, in total, eight protein extracts were analyzed: four from parasites not exposed to PZQ (NEPZQ) [resistant males and females (assigned as RM-NEPZQ and RF-NEPZQ, respectively), and susceptible males and females (assigned as SM-NEPZQ and SF-NEPZQ, respectively)] and another four samples from parasites exposed to PZQ (EPZQ) [resistant males and females (assigned as RM-EPZQ and RF-EPZQ, respectively), and susceptible males and females (assigned as SM-EPZQ and SF-EPZQ, respectively)]. All protein extracts presented high purity and good quality for posterior 2-DE and mass spectrometry (MS) analysis (Figure IV-1).

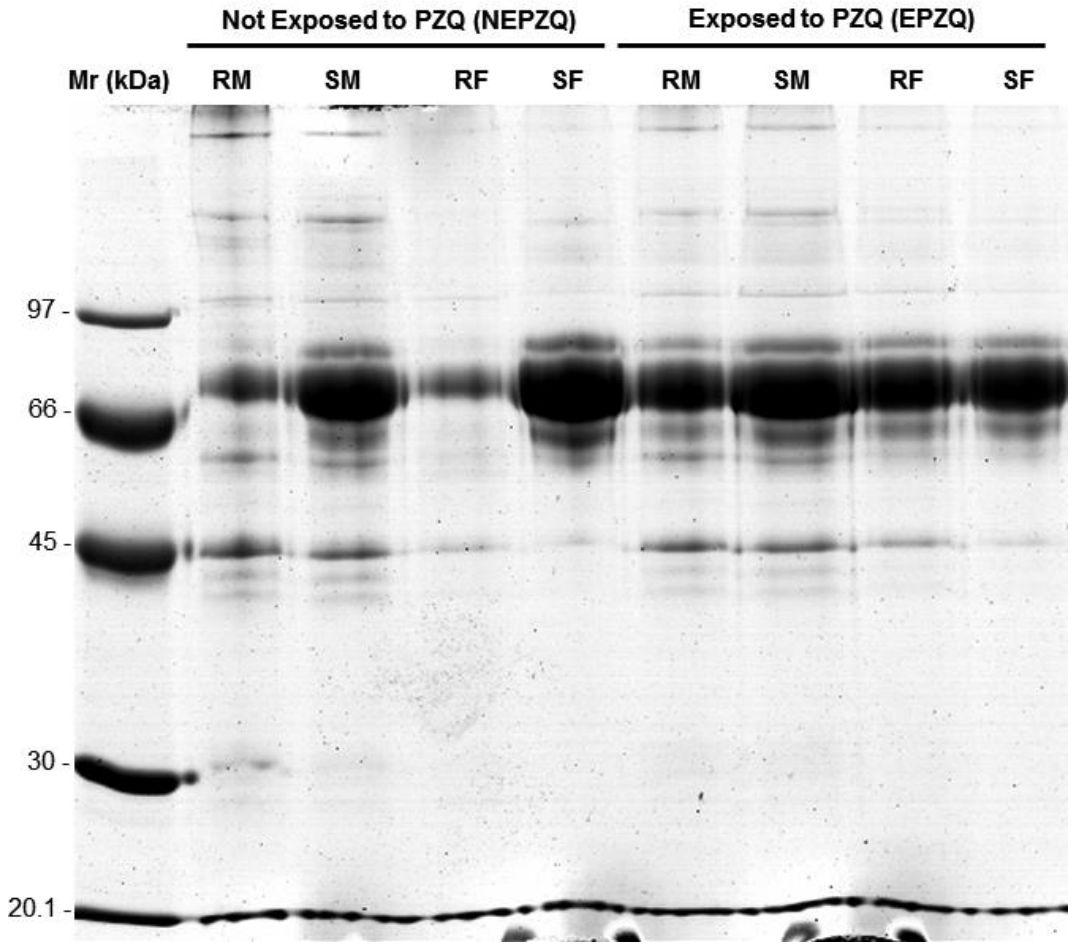

Figure IV-1. SDS-PAGE gel of the protein preparations, confirming the quality of the protein extracts studied. RM – Resistant males; SM – Susceptible males; RF – Resistant females; SF – Susceptible females; Mr – Molecular reference.

Analytical 2-DE gels were produced using 13 cm, pH 3-10NL strips and SDS-PAGE 12%, stained by Coomassie Blue to reproducibly resolve protein spots in a broad pH range and molecular weight, and posteriorly compare the protein pattern of *S. mansoni* proteome from the two strains (PZQ-resistant and PZQ-susceptible) not exposed (Figure IV-2) and exposed to PZQ (Figure IV-3).

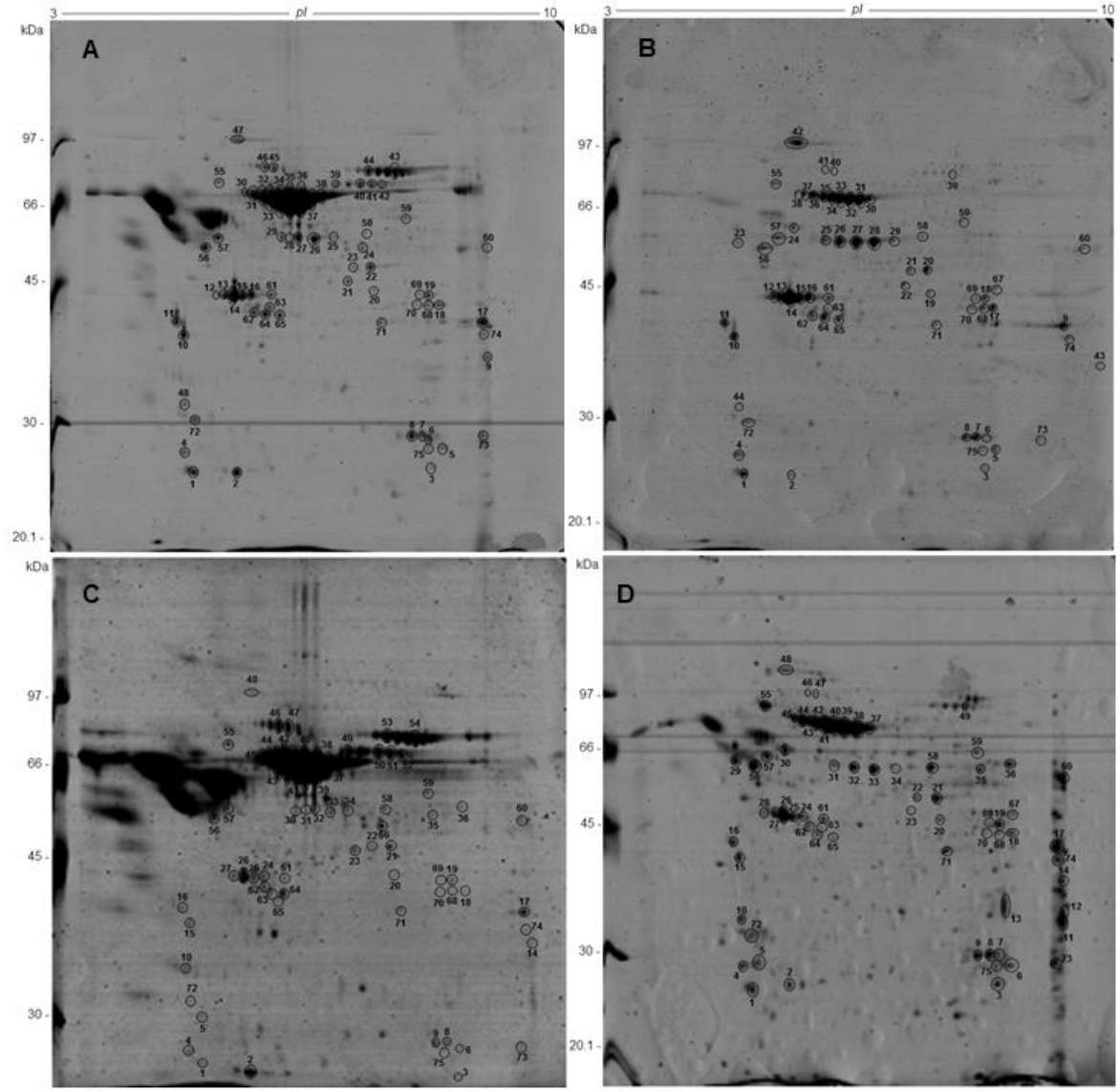

Figure IV-2. Two-dimensional gel electrophoresis of protein samples from *S. mansoni* adult worms not exposed to PZQ using 13 cm, pH 3-10NL strips and SDS-PAGE 12%, stained by Coomassie Blue. A - SM-NEPZQ; B – RM-NEPZQ; C – SF-NEPZQ; D – RF-NEPZQ. Numbers identify the spots, which were analyzed and identified by MS. All the identified proteins are listed in Table IV-2. The figure shows one representative experiment of three replicates.

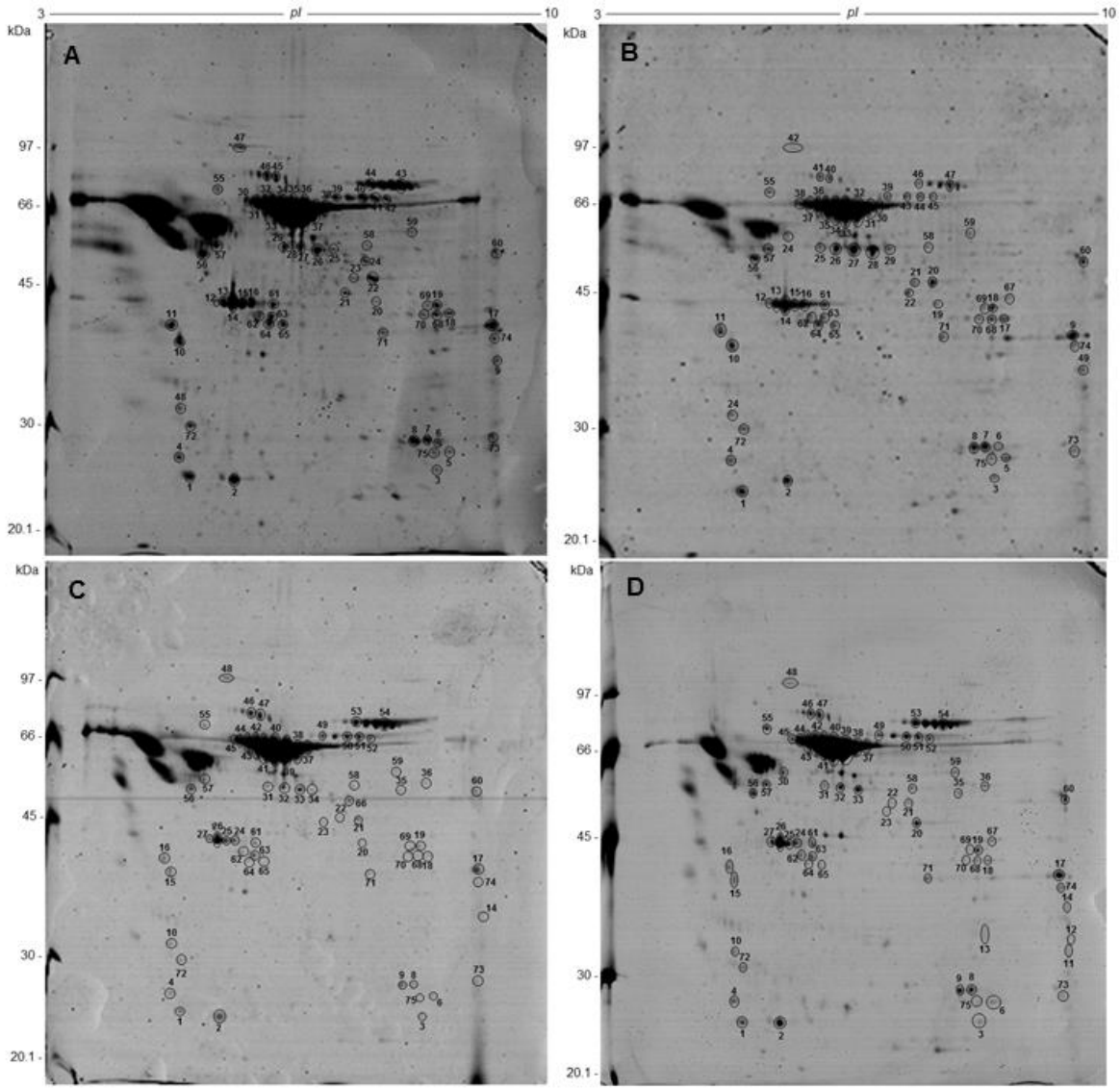

Figure IV-3. Two-dimensional gel electrophoresis of protein samples from *S. mansoni* adult worms exposed to PZQ using 13 cm, pH 3-10NL strips and SDS-PAGE 12%, stained by Coomassie Blue. A - SM-EPZQ; B – RM-EPZQ; C – SF-EPZQ; D – RF-EPZQ. Numbers identify the spots, which were analyzed and identified by MS. All the identified proteins are listed in Table IV-2. The figure shows one representative experiment of three replicates.

2-DE maps constructed with Coomassie Blue-stained gels showed reasonably comparable numbers of spots in all the samples. In total 133 ± 14, 265 ± 20, 142 ± 8 and 188 ± 34 spots were detected in proteins from RM-NEPZQ, RF-NEPZQ, SM-NEPZQ and SF-NEPZQ, respectively (Table IV-1). For parasites exposed to PZQ, 203 ± 14, 133 ± 9, 220 ± 34 and 99 ± 19 spots were detected in RM-EPZQ, RF-EPZQ, SM-EPZQ and SF-EPZQ, respectively (Table IV-1). It is worth noting that 2-DE gels from RF-NEPZQ, RM-EPZQ and SM-EPZQ contain larger numbers of protein spots compared to other samples.

Table IV-1. Summary comparison of the number of protein spots in the 2-DE maps for the eight different protein extracts analyzed.

	Not exposed to PZQ (NEPZQ)				Exposed to PZQ (EPZQ)			
Samples	RM	RF	SM	SF	RM	RF	SM	SF
Replica 1	145	243	151	150	215	124	242	80
Replica 2	135	270	138	200	188	135	181	99
Replica 3	118	281	136	215	207	141	237	117
Mean ± SD	133 ± 14	265 ± 20	142 ± 8.0	188 ± 34	203 ± 14	133 ± 9.0	220 ± 34	99 ± 19.0

4.2. LC-MS/MS analysis and protein identification

The spots differentially expressed were excised from preparative gels of each sample, digested by Trypsin and identified by LC-MS/MS. For RM-NEPZQ 64 spots were successfully analyzed by LC-MS/MS, as well as 69 from RF-NEPZQ, 67 from SM-NEPZQ, 68 from SF-NEPZQ, 68 from RM-EPZQ, 69 from RF-EPZQ, 67 from SM-EPZQ, and 66 spots from SF-EPZQ (Table IV-2). The MS/MS results were employed to search the database (NCBInr) by Mascot search engine, and the matched proteins are listed in Table IV-2. Some proteins were identified in only one individual spot, but on several occasions, more than one spot in a gel corresponded to the same protein or better, same isoforms (Figure IV-2, Figure IV-3 and Table IV-2).

Table IV-2. Proteins and spots identified in the samples from parasites not exposed and exposed to PZQ.

Protein Description	Accession	Gene ID	Mw	pI	Spot number (Not exposed to PZQ)				Spot number (Exposed to PZQ)			
					SM	RM	SF	RF	SM	RM	SF	RF
14-3-3 epsilon isoform	Q9U491	Smp_034840.3	28.850	4.85	72	72	72	72	72	72	72	72
14-3-3 protein homolog 1	Q26540	N/A	28.468	4.74	4	4	4	4	4	4	4	4
14-3-3 protein homolog 2	Q26537	N/A	24.706	4.98	2	2	2	2	2	2	2	2
Actin-1	P53470	N/A	41.990	5.3	13, 14, 15, 16, 19	13, 14, 15, 16, 18	19, 24, 25, 26, 27	19, 24, 25, 26, 27	13, 14, 15, 16, 19	13, 14, 15, 16, 18	19, 24, 25, 26, 27	19, 24, 25, 26, 27
Actin-2	P53471	N/A	41.999	5.3	13, 14, 15, 16	13, 14, 15, 16	24, 25, 26, 27	24, 25, 26, 27	13, 14, 15, 16	13, 14, 15, 16	24, 25, 26, 27	24, 25, 26, 27
Albumin	Q95VB7	N/A	70.177	5.97	30, 31, 33, 34, 35, 36, 37, 38	30, 31, 32, 33, 34, 35, 37, 38	37, 38, 39, 40, 41, 42, 44, 45	37, 38, 39, 40, 41, 42, 44, 45	30, 31, 33, 34, 35, 36, 37, 38	30, 31, 32, 33, 34, 35, 37, 38	37, 38, 39, 40, 41, 42, 44, 45	37, 38, 39, 40, 41, 42, 44, 45
Aldehyde dehydrogenase	G4LWI3	Smp_050390	54.412	5.76	25, 26, 27, 28, 29	25, 26, 27, 28, 29	30, 31, 32, 33, 34	31, 32, 33, 34	25, 26, 27, 28, 29	25, 26, 27, 28, 29	30, 31, 32, 33, 34	31, 32, 33, 34
Alpha tubulin	Q26595	sat1	50.660	4.97	57	57	57	57	57	57	57	57
ATP-dependent transporter	G4V904	Smp_049500	82.249	6.32	40		50		40	43	50	50
Beta1,3-galactosyltransferase	G4VBF6	Smp_102400	41.050	8.76				12				12
Calponin	P91888	N/A	38.504	9.26	9	43	14	14	9	49	14	14
Cathepsin L	Q26564	N/A	37.004	8.58				11				11
Cell polarity protein	G4LY48	Smp_146240	64.048	6.97	20	19	20	20	20	19	20	20
Collagen alpha chain	G4VIR7	Smp_119050	23.687	5.01			5	5				
Cytosol aminopeptidase	P91803	N/A	56.897	7.56			36	36			36	36
DNA helicase	G4M028	Smp_094140	94.407	5.86	45	40	47	47	45	40	47	47
Dopamine transporter	E9LD23	Slc6a3	83.701	6.82		39		49				
Elongation factor 1-alpha	G4VAD2	Smp_099870	51.348	8.94	60	60	60	60	60	60	60	60
Enolase	Q27877	ENO	47.421	6.18	22, 23	20, 21	21, 22	21, 22	22, 23	20, 21	21, 22	21, 22
Fructose bisphosphate aldolase	P53442	N/A	39.963	7.63	18, 68, 70	17, 68, 70	18, 68, 70	18, 68, 70	18, 68, 70	17, 68, 70	18, 68, 70	18, 68, 70

Table IV-2 (Continuation 1). Proteins and spots identified in the samples from parasites not exposed and exposed to PZQ.

Protein Description	Accession	Gene ID	Mw	pI	Spot number (Not exposed to PZQ)				Spot number (Exposed to PZQ)			
					SM	RM	SF	RF	SM	RM	SF	RF
Galactokinase	G4VRV4	Smp_078400	55.038	5.75		24		30		24		30
Gelsolin	G4VIJ2	Smp_197860	40.373	6.38	21, 61	22, 61	23, 61	23, 61	21, 61	22, 61	23, 61	23, 61
Glutathione S-transferase 28 kDa (GST-28)	P09792	Smp_054160	23.861	6.56	7, 8	7, 8	8, 9	8, 9	7, 8	7, 8	8, 9	8, 9
Glyceraldehyde-3-phosphate dehydrogenase	P20287	N/A	36.640	8.16	17	9	17	17	17	9	17	17
Heat shock 70 kDa protein homolog	P08418	N/A	68.331	5.4	31, 32	35, 36, 37	43, 44	42, 43, 44	31, 32	35, 36, 37	43, 44	42, 43, 44
Heat shock protein 70 (HSP 70)	G4V910	Smp_049550	71.479	5.12	55	55	55	55	55	55	55	55
Inosine-5'-monophosphate dehydrogenase	G4V9I5	Smp_012930	55.371	7.62			36	36			36	36
Lysine tRNA ligase	G4M0E0	Smp_104470.1	72.099	6.15	43		54		43	47	54	54
Major egg antigen (p40)	P12812	N/A	39.575	6.23	71	71	71	71	71	71	71	71
Malate dehydrogenase	G4VBJ0	Smp_047370	36.761	8.7	74	74	74	74	74	74	74	74
Myosin regulatory light chain	G4LUD9	Smp_132670.1	22.705	4.88	1	1	1	1	1	1	1	1
Ornithine aminotransferase	A7UAX6	N/A	49.189	8.83		67		67		67		67
Paramyosin	P06198	N/A	100.38	5.31	47	42	48	48	47	42	48	48
Phosphoglycerate kinase	P41759	PGK	44.879	6.84	69	69	69	69	69	69	69	69
Phosphoglycerate mutase	G4VJD5	Smp_096760	28.579	7.71	73	73	73	73	73	73	73	73
Phosphopyruvate hydratase	G4VQ58	Smp_024110	47.433	6.18	24		66		24		66	
Protein disulfide-isomerase ER-60	P38658	N/A	54.785	6.3	58, 59	58, 59	58, 59	58, 59	58, 59	58, 59	58, 59	58, 59
Protein kinase	G4LW63	Smp_131800	69.652	5.25	41		51		41	44	51	51
Rab6-interacting protein 2 (ERC protein 1)	G4V9A7	Smp_059780	57.834	4.7		24		30		24		30
Receptor for activated Protein Kinase C (PKC)	Q8T6T3	Smp_102040.1	35.208	7.12				13				13
RNA m5u methyltransferase	G4VF51	Smp_123520	69.936	5.93	44		53		44	46	53	53
Serine/threonine kinase	G4V6Z8	Smp_162010	94.088	9.47	46	41	46	46	46	41	46	46
Serine/threonine phosphatase	Q3ZFF5	N/A	38.040	6.34	12	12		28	12	12		
Sorting Nexin-related	G4VRE2	Smp_042550	49.693	5.6	20	19	20	20	20	19	20	20
Suppressor of actin (Sac)	G4VJY5	Smp_060420.2	63.462	7.01	42		52		42	45	52	52
Tegument antigen (Antigen SmA22.6)	P14202	A12	22.848	6.63	3	3	3	3	3	3	3	3

Table IV-2 (Continuation 2). Proteins and spots identified in the samples from parasites not exposed and exposed to PZQ.

Protein Description	Accession	Gene ID	Mw	pI	Spot number (Not exposed to PZQ)				Spot number (Exposed to PZQ)			
					SM	RM	SF	RF	SM	RM	SF	RF
Transducin beta-like	G4M196	Smp_098760	75.243	5.88		35		42		35		42
Triosephosphate isomerase	P48501	TPI	28.447	7.64	5, 75	5, 75	6, 75	6, 75	5, 75	5, 75	6, 75	6, 75
Tropomyosin-1	P42637	N/A	32.991	4.62	11	11	16	16	11	11	16	16
Tropomyosin-2	P42638	N/A	32.676	4.5	10	10	15	15	10	10	15	15
Troponin I	G4VGQ7	Smp_018250.1	26.259	9.45	6	6		7	6	6		
Troponin T	G4LYF7	Smp_179810	37.472	5.89	62, 63, 64, 65	62, 63, 64, 65	62, 63, 64, 65	62, 63, 64, 65	62, 63, 64, 65	62, 63, 64, 65	62, 63, 64, 65	62, 63, 64, 65
Tubulin beta chain	C4QIC0	Smp_078040	50.245	4.79	56	56	56	56	56	56	56	56
Twister (putative)	G4VCM6	Smp_147290	59.054	4.88		23		29				
Ubiquitin protein ligase E3a	G4VAP0	Smp_172420	347.14	8.72			35	35			35	35
Ubiquitin-specific peptidase 30 (C19 family)	G4VCG9	Smp_122960.1	69.295	6.92		39		49				
Uncharacterized protein, Smp_018790 (91.6% identity with PP2C-like domain-containing protein - *S. haematobium*	G4M036	Smp_018790	78.184	6.09	40		50		40	43	50	50
Uncharacterized protein, Smp_161260 (63.4% identity with SJCHGC05745 protein - *S. japonicum*	G4M0B0	Smp_161260	84.962	5.69	39		49		39	39	49	49
Uncharacterized protein, Smp_162220 (88% identity with SJCHGC07938 protein - *S. japonicum*)	G4VLB0	Smp_162220	95.145	6.8		39		49				
Uncharacterized protein, Smp_171780 (95.2% identity with SPARC - *S. haematobium*	G4VJ77	Smp_171780	30.071	4.79	48	44	10	10	48	24	10	10
Number of spots identified					**67**	**64**	**68**	**69**	**67**	**68**	**66**	**69**
Number of proteins identified					**45**	**45**	**47**	**52**	**45**	**48**	**46**	**52**

Sixty individual protein species were identified on samples from parasites not exposed to PZQ, of which 45 were present in RM-NEPZQ, 52 in RF-NEPZQ, 45 in SM-NEPZQ and 47 proteins in SF-NEPZQ (Table IV-2). In this group of NEPZQ parasites, 35 proteins were common to all the four protein extracts, eight occurred only in resistant worms, eight only in susceptible worms, four in female parasites, and three were only present in resistant females. Interestingly, two proteins that have shown to be common in RM-NEPZQ, RF-NEPZQ and SM-NEPZQ preparations, namely Serine/Threonine phosphatase and Troponin I, did not appear in SF-NEPZQ (Figure IV-4, Figure IV-5A and Table IV-3).

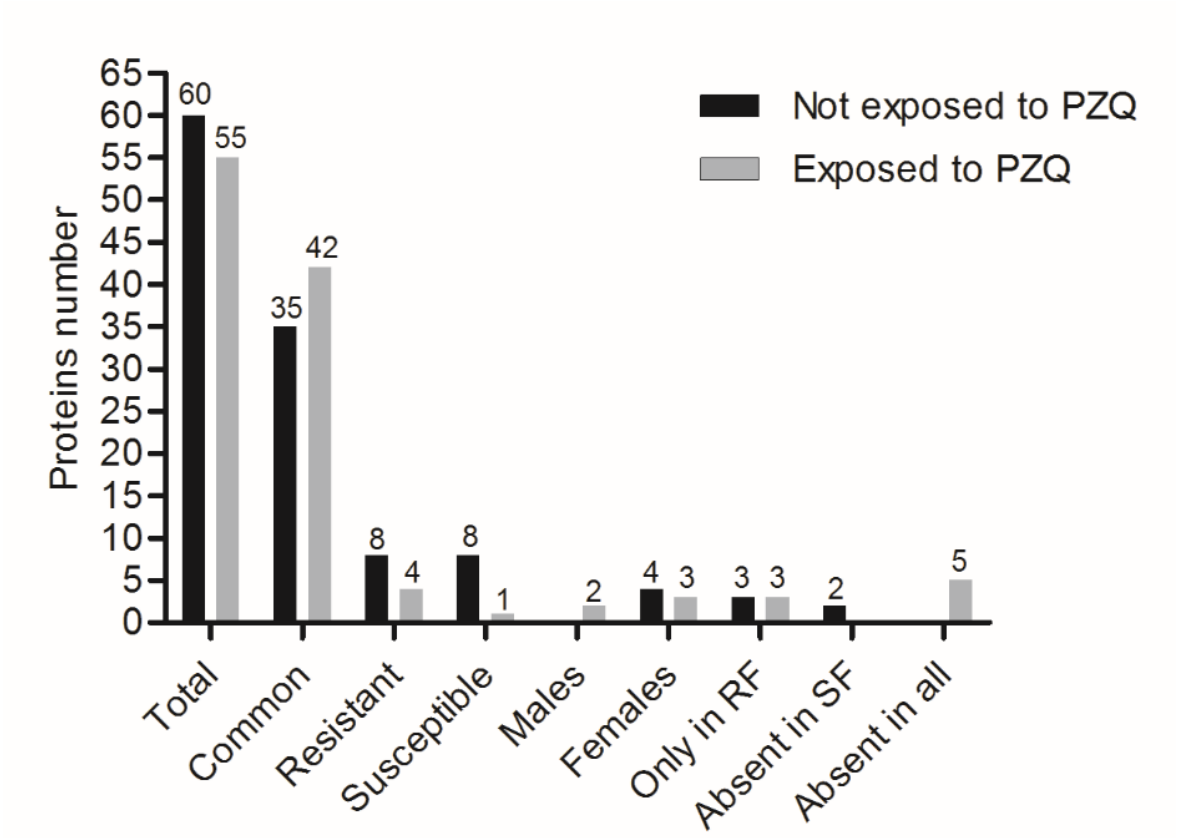

Figure IV-4. Number of unique and shared proteins identified between and among the protein preparations from parasites not exposed and exposed to PZQ.

The total number of proteins identified was reduced to 55 proteins on the protein extracts from parasites exposed to PZQ. Forty-eight of those proteins were present in RM-EPZQ, 52 in RF-EPZQ, 45 in SM-EPZQ, and 46 in SF-EPZQ (Table IV-2). Forty-two proteins appeared to be common to all the four proteins preparations of EPZQ parasites. In addition, four proteins were present only in resistant strain, one in susceptible strain, three were exclusive of female parasites, two proteins were present only in male parasites and three were only in resistant females. Five proteins that were present on parasites not exposed to PZQ, namely, collagen alpha chain, dopamine transporter, twister (putative), ubiquitin-specific peptidase 30 (C19 family), and Smp_162220, did not appear here (Figure IV-4, Figure IV-5B and Table IV-3).

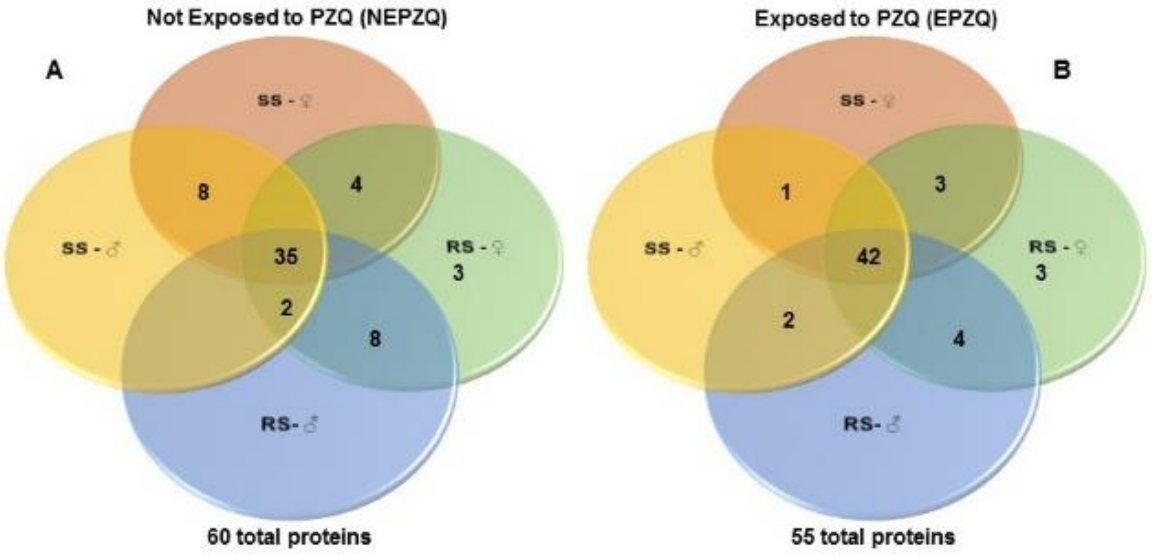

Figure IV-5. Venn diagram showing the number shared proteins identified between and among the protein preparations from parasites. A) Not Exposed to PZQ; B) Exposed to PZQ. RS-♂: resistant strain males; RS-♀: resistant strain females; SS-♂: susceptible strain males; SS-♀: susceptible strain females. Common spots identified between and among the samples are represented overlapped by the circles.

Table IV-3. Specific proteins identified in each group analyzed.

Worms	Not exposed to PZQ (NEPZQ)		Exposed to PZQ (EPZQ)	
	Resistant strain	Susceptible strain	Resistant strain	Susceptible strain
Males			-Serine / threonine phosphatase; -Troponin I.	-Serine / threonine phosphatase; -Troponin I.
Females	-Collagen alpha chain; -Cytosol aminopeptidase; -Inosine-5'-monophosphate dehydrogenase; -Ubiquitin protein ligase E3a; *- Beta 1,3-galactosyltransferase; *- Cathepsin L; *- Receptor for activated Protein Kinase C (PKC).	-Collagen alpha chain; -Cytosol aminopeptidase; -Inosine-5'-monophosphate dehydrogenase; -Ubiquitin protein ligase E3a;	-Cytosol aminopeptidase; -Inosine-5'-monophosphate dehydrogenase; -Ubiquitin protein ligase E3a; *- Beta 1,3-galactosyltransferase; *- Cathepsin L; *- Receptor for activated Protein Kinase C (PKC).	-Cytosol aminopeptidase; -Inosine-5'-monophosphate dehydrogenase; -Ubiquitin protein ligase E3a;
Both	-Galactokinase; -Ornithine aminotransferase; -Rab6-interacting protein 2 (ERC protein 1); -Transducin beta-like; -Dopamine transporter; -Twister, putative; -Ubiquitin-specific peptidase 30 (C19 family); -Smp_162220.	-ATP-dependent transporter; -Lysine tRNA ligase; -Phosphopyruvate hydratase; -Protein kinase; -RNA m5u methyltransferase; -Suppressor of actin (Sac); -Smp_018790; -Smp_161260.	-Galactokinase; -Ornithine aminotransferase; -Rab6-interacting protein 2 (ERC protein 1); -Transducin beta-like.	-Phosphopyruvate hydratase.

*****Proteins present only in resistant females.

4.3. Molecular function of identified proteins

The proteins identified by MS/MS, and summarized in Table IV-2, were categorized by their molecular function, according to information obtained from the GO database (Table IV-4), as described in the Material and Methods section. When proteins had another function annotation, they were shown in brackets. The biological process and subcellular localization assigned to each protein in that database are also included in the Table IV-4. The results allowed the identification of proteins categorized as binding, catalytic, transport, regulation of muscle contraction, chaperone, motor, structural activities and proteins of unknown functions. Among the molecules identified as

binding proteins, most of them were ATP, nucleotide, protein and ion binding proteins. The proteins categorized correspond to a variety of biological processes, nevertheless most of them were glycolytic enzymes and proteins related to metabolic process.

Regarding to the subcellular localization, the proteins identified were classified as cytoskeletal, cytosolic, nuclear, membrane proteins and some of them were located on extracellular matrix. Among them, the most abundantly identified were cytosolic proteins. There were fifteen and seven proteins whose biological process and subcellular localization, respectively, were not predicted (Table IV-4).

Table IV-4. Proteins identified by their MS/MS and categorized by their molecular function according to information obtained from GO database.

Molecular function	Protein name	Biological Process	Subcellular localization
Binding (19)	-14-3-3 epsilon	-	-Cytosol
	-14-3-3 protein homolog 1	-	-Cytosol
	-14-3-3 protein homolog 2	-	-Cytosol
	-Actin-1 **(Motor, structural)**	-	-Cytoskeleton
	-Actin-2 **(Motor, structural)**	-	-Cytoskeleton
	-Calponin	-Actomyosin structure organization	-Cytosol
	-Cytosol aminopeptidase	-Metabolic process; proteolysis	-Cytosol
	-DNA helicase	-DNA replication	-Nucleus
	-Dopamine transporter **(Transport)**	-Transport	-Membrane
	-Gelsolin	-	-Cytoskeleton
	-Heat shock 70 kDa protein homolog **(Chaperone)**	-Stress response	-Cytosol
	-Heat shock protein 70 (HSP 70) **(Chaperone)**	-Stress response	-Cytosol
	-Myosin regulatory light chain	-Muscle contraction	-Cytoskeleton
	-Receptor for activated Protein Kinase C (PKC)	-	-
	-Sortingnexin-related	-Endocytosis	-Membrane
	-Tegument antigen (Antigen SmA22.6)	-Microtubule-based process	-Membrane
	-Troponin I	-	-Cytosol
	-Uncharacterized protein, Smp_162220 (88% identity with SJCHGC07938 protein - *S. japonicum*)	-	-
	-Uncharacterized protein, Smp_171780 (95,2% identity with SPARC protein - *S. haematobium*)	-Signal transduction	-Extracellular matrix

*****Numbers in brackets indicate the amount of proteins with those certain functions.

Table IV-4 (Continuation 1). Proteins identified by their MS/MS and categorized by their molecular function according to information obtained from GO database.

Molecular function	Protein name	Biological Process	Subcellular localization
Catalytic activity (15)	-Aldehyde dehydrogenase	-Metabolic process	-Cytosol
	-Beta 1,3-galactosyltransferase	-Protein glycosylation	-Membrane
	-Cathepsin L	-Proteolysis	-
	-Fructose bisphosphate aldolase	-Glycolysis	-Cytosol
	-Glutathione S-transferase 28 kDa	-Detoxification	-Cytosol
	-Malate dehydrogenase	-Metabolic process	-Cytosol
	-Phosphoglycerate mutase	-Glycolysis	-Cytosol
	-Protein disulfide-isomerase ER-60	-Metabolic process	-Cytosol
	-RNA m5u methyltransferase	-Methylation	-Cytosol
	-Suppressor of actin (Sac)	-Metabolic process	-Membrane
	-Transducin beta-like	-Regulation of transcription	-Cytosol
	-Triosephosphate isomerase	-Glycolysis	-Cytosol
	-Ubiquitin protein ligase E3a	-Protein ubiquitination	-Cytosol
	-Ubiquitin-specific peptidase 30 (C19 family)	-Protein ubiquitination	-Cytosol
	-Uncharacterized protein, Smp_018790 (91,6% identity with PP2C-like domain-containing protein - *S. haematobium*)	-Metabolic process	-Cytosol
Binding and Catalytic activity (16)	-Alpha tubulin	-Microtubule-based process	-Cytoskeleton
	-ATP-dependent transporter **(Transport)**	-Metabolic process	-Membrane
	-Cell polarity protein	-Metabolic process	-Membrane
	-Elongation factor 1-alpha	-Protein biosynthesis	-Cytosol
	-Enolase	-Glycolysis	-Cytosol
	-Galactokinase	-Metabolic process	-Cytosol
	-Glyceraldehyde-3-phosphate dehydrogenase	-Glycolysis	-Cytosol
	-Inosine-5'-monophosphate dehydrogenase	-Protein biosynthesis	-Cytosol
	-Lysine tRNA ligase	-Protein biosynthesis	-Cytosol
	-Ornithine aminotransferase	-Metabolic process	-Cytosol
	-Phosphoglycerate kinase	-Glycolysis	-Cytosol
	-Phosphopyruvate hydratase	-Glycolysis	-Cytosol
	-Protein kinase	-Phosphorylation	-Cytosol
	-Serine/threonine kinase	-Phosphorylation	-Cytosol
	-Serine/threonine phosphatase	-Metabolic process and dephosphorylation	-Cytosol
	-Tubulin beta chain	-Microtubule-based process	-Cytoskeleton

*****Numbers in brackets indicate the amount of proteins with those certain functions.

Table IV-4 (Continuation 2). Proteins identified by their MS/MS and categorized by their molecular function according to information obtained from GO database.

Molecular function	Protein name	Biological Process	Subcellular localization
Transport (4)	-Albumin	-Transport	-Extracellular matrix
	-Rab6-interacting protein 2 (ERC protein 1)	-	-
Regulation of muscle contraction (4)	-Tropomyosin-1	-Muscle contraction	-Cytosol
	-Tropomyosin-2	-Muscle contraction	-Cytosol
	-Troponin I	-	-Cytosol
	-Troponin T	-	-Cytosol
Chaperone (3)	-Major egg antigen (p40)	-	-Cytosol
Motor (3)	-Paramyosin	-Muscle contraction	-Cytoskeleton
Structural (3)	-Collagen alpha chain	-	-
Unknown (2)	-Twister (putative)	-	-
	-Uncharacterized protein, Smp_161260 (63,4% identity with SJCHGC05745 protein - *S. japonicum*)	-	-

*****Numbers in brackets indicate the amount of proteins with those certain functions.

5. Discussion

Schistosomiasis is one of the most important infectious parasitic diseases, affecting millions of people worldwide, and representing a serious health problem [1, 2, 45]. Nowadays its control is based on PZQ, the only drug available for its treatment, which heavily relies on massive chemotherapy [3, 5, 7] However, the report of PZQ-resistance cases by *S. mansoni* has become a serious problem that needs to be solved. Several reports from our group and others have suggested that resistance to *Schistosoma* infection can be acquired naturally or induced by drug [37, 46-48]. Besides experimental evidences, reports of treatment failure in Senegal and Egypt in isolates with reduced susceptibility to PZQ were obtained [49, 50] and further *ex vivo* experiments have confirmed the development of PZQ-resistance [37, 51-54]. Our group in particular has shown that resistance may be developed by drug pressure and as discussed in the Introduction section of this Chapter, we developed an important model that allows the laboratory study of PZQ-resistance in *S. mansoni* [37]. Taking advantage of our PZQ-resistant strain, the present study represents the first report of a *S. mansoni* PZQ-resistant strain proteomics analysis, comparing this strain with its isogenic counterpart susceptible strain, which is different only for the genetic determinants accounting for the PZQ-resistance phenotype.

In the present study we identified 60 different proteins on *S. mansoni* proteome. All those proteins were present in worms not exposed to PZQ, but some of them disappeared when these worms were exposed to the same drug. This result could possibly indicate an effect of PZQ exposure on protein expression in resistant and susceptible strains. Although previous studies of *Schistosoma* proteome had been performed using protein extracts and *Schistosoma* species different from ours, some proteins, such as 14-3-3 protein, HSP-70, GAPDH, glutathione S-transferase 28 kDa, enolase, fructose-bisphosphate aldolase, actin, triose phosphate isomerase, calponin, elongation factor 1-α, phosphoglycerate kinase, phosphoglycerate mutase, myosin, and paramyosin were commonly identified [29, 36, 55-61]. In addition, some proteins that have already been tested as vaccine candidates, as glutathione S-transferase 28 kDa [62, 63], triose phosphate isomerase [63, 64] and paramyosin [63] were also identified in the present study. Major egg antigen, troponin T, disulphide-isomerase ER-60 and actin, proteins that we also found, have already been clustered as

immunoreactive proteins in serum pools of infected or non-infected individuals from endemic area [36].

Looking at the proteomes from both genders, in this survey, four proteins were only expressed in females from both strains, even under exposure to PZQ, namely, cytosol aminopeptidase, inosine-5'-monophosphate dehydrogenase (IMPDH), ubiquitin protein ligase E3a, and collagen alpha chain. Cytosol/leucine aminopeptidase catalyzes the hydrolysis of amino-acid residues from N-terminus of proteins and peptides [65], and it has already been assessed as a vaccine candidate against the infection of *Fasciola hepatica* [66]. This protein has previously been identified in *S. mansoni* eggs [67]. Regarding IMPDH, this protein is responsible for the rate-limiting step in guanine nucleotide biosynthesis [68], and it has previously been identified in *Schistosoma* genome and transcriptome [69]. E3 ligase enzyme catalyzes protein ubiquitination, which regulates various biological processes through covalent modification of proteins and transcription factors, and ubiquitin is the most important protein of this process [70, 71]. It has been suggested that ubiquitination is of interest in *S. mansoni* because this process could be a potential target for the design of new drugs [72], being ubiquitin protein ligase E3a a good target to be studied. In regard to collagen alpha chain, Yang and colleagues [73] described that silencing the expression of a type of collagen (type V collagen) significantly affects the spawning and egg hatching of *S. japonicum*, and it also affects the morphology of the worms [73]. Therefore, it would be very interesting evaluate the role of each of these proteins in PZQ-resistance, specially, collagen alpha chain, since it seems to occur morphological alterations in eggs and worms of *S. mansoni* PZQ-resistant strain [74]. Moreover, females of this species, the only gender in which those proteins were found in this work, are more tolerant to PZQ treatment than males [37].

Another large difference between the proteome of both genders was the expression of troponin I and serine/threonine-protein phosphatase. Those proteins were present in males independently of drug exposure, but in females they were only present in resistant females not exposed to PZQ. Troponin I belongs to the troponin complex that mediate Ca^{2+}-regulation that governs the actin-activated myosin motor function in striated muscle contraction [75]. On the other side, protein kinases and phosphatases, as is the case of serine/threonine-protein phosphatase, are essential for normal functioning of signaling pathways, since it is well known that reversible phosphorylation

of proteins is a ubiquitous mechanism crucial for regulation of most cellular functions [76]. In *S. mansoni*, a phosphatase 2B (calcineurin) has been described as a heterodimer with a catalytic subunit and a regulatory subunit, which bind to Ca^{2+} increasing the phosphatase activity [77, 78]. Thus, protein phosphatases represent crucial molecules for the parasite and hence potential chemotherapeutic targets [79]. Those differences in proteomes of males and females of *S. mansoni* represents a remarkable finding that is in agreement with other works [59, 80], who have described a differential protein expression between males and females of *Schistosoma* spp.. However, these proteins are different from those described here, but it should be taken into account that those studies were performed with different species of *Schistosoma* [59, 80].

Regarding to resistant strain parasites, it is notable the finding of eight proteins exclusively expressed in those *S. mansoni* worms. From those eight proteins, dopamine transporter, twister (putative), ubiquitin-specific peptidase 30 (C19 family), and uncharacterized protein smp_162220 are not present in *S. mansoni* PZQ-exposed worms. However, galactokinase, ornithine aminotransferase, Rab6-interacting protein 2 (ERC protein 1) and transducin beta-like remained after drug exposure. Dopamine/norepinephrine transporter (*SmDAT*) gene transcript, characterized in *S. mansoni*, is essential for the survival of the parasite as it causes muscular relaxation and a lengthening in the parasite, controlling movement [81]. Galactokinase catalyzes the second step of the Leloir pathway, a metabolic pathway found in most organisms for the catabolism of β-D-galactose to glucose 1-phosphate [82]. Galactokinase and hexokinase have similar enzymatic function on sugar phosphorylation [83], and characterization of schistosome hexokinase has been described as pertinent to understanding the metabolic response of *S. mansoni* cercariae to an increased glucose availability [84]. Ornithine aminotransferase was already identified in *S. mansoni* [85] and it has been characterized as playing a central role in ornithine biosynthesis [86]. It seems responsible for catalyzing the transfer of the delta-amino group of L-ornithine to 2-oxoglutarate, producing L-glutamate-gamma-semialdehyde, that in turn spontaneously cyclizes to pyrroline-5-carboxylate, and L-glutamate [87]. Rab6-interacting protein 2 is a member of a family of RIM-binding proteins, which are presynaptic active zone proteins that regulate neurotransmitter release [88]. Ubiquitin-specific peptidase 30 (C19 family) belongs to a metabolic pathway that had previously

been associated to development of artemisinin and artesunate-resistance in *Plasmodium chabaudi* [89], which is a very interesting result. All those proteins specifically found in the resistant strain should be further studied to better understand if they could possibly have a fundamental role in PZQ-resistance development.

Yet for the resistant strain parasites, there are three proteins, beta 1,3-galactosyltransferase, cathepsin L, and receptor for activated Protein Kinase C (PKC) that are exclusive to resistant females, even after exposure to PZQ. Beta 1,3-galactosyltransferase has previously been identified in *Schistosoma* genome and transcriptome [69]. Cathepsin L activity is believed to be involved in hemoglobin digestion by adult schistosomes [90], and Dalton and colleagues [90] suggested the involvement of cathepsin proteinases in several key functions render them as potential targets to novel antiparasitic chemotherapy and immunoprophylaxis. Putative PKC exists in kinomes of the blood flukes *S. mansoni* [91, 92], *S. japonicum* [93] and *S. haematobium* [94], and regulates movement, attachment, pairing, and egg release in *S. mansoni*, being considered a potential target for chemotherapeutic treatment against schistosomiasis [95]. These results really suggest a possible relationship between those proteins and PZQ-resistance in *S. mansoni* females, possibly being responsible for the exacerbated resistance demonstrated by those females in previous work performed by us [37].

Concerning the PZQ-susceptible strain, it should be noted eight proteins that only appeared in this strain. Phosphopyruvate hydratase (enolase), an important glycolytic enzyme that has the functions of activating the plasminogen, involving in the processes of infection and migration of parasites, reducing the immune function of the host as well as preventing parasites from the immune attack of the host [96], is the only protein from those eight proteins that continued to be expressed after PZQ exposure. This possibly suggests a relationship of this protein with the PZQ-susceptibility/resistance. However, more studies are necessary to investigate this hypothesis.

All those results together represent an important finding for the study of PZQ-resistance/susceptibility in *S. mansoni*, since they allow comparing directly the proteome under both conditions. We believe that the most promising candidates are proteins that appeared associated only to one of the strains, especially those with functions possibly related with the phenotypic alterations observed in the Chapter III

[74], or previously associated with resistance by others parasites to different drugs. These candidates require special attention in more studies, assessing for instance the level of protection induced by these proteins in animal models infected by both *S. mansoni* strains, as they may have some involvement on PZQ-resistance phenomenon.

In conclusion, due to the increase of resistance by *S. mansoni* worms to the only drug available to treat schistosomiasis, it is imperative and urgent to study this phenomenon trying to understand what is involved in its occurrence. In this context, this work is extremely relevant since for the first time the proteome of a *S. mansoni* PZQ-resistant strain is characterized and compared to the proteome of *S. mansoni* PZQ-susceptible strain. The proteomics approach conducted here has lead to the identification of several proteins that were found to be characteristic of PZQ-resistant strain, and could putatively be involved in the PZQ-resistance phenomenon. Therefore, this is an innovative study that opens doors to PZQ-resistance surveys, contributing to discover a solution to PZQ-resistance problem as suggest new potential targets for new drugs.

6. References

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CHAPTER V – FINAL DICUSSION

V. Final discussion and future directions

1. Final discussion and future directions

The emergence of PZQ-resistance in *Schistosoma* spp. populations is increasingly considered as a fact, and continued vigilance is recommended by health authorities [1, 2]. Since EPs are involved in several chemotherapy failures [3, 4], in this work we tested their involvement in the PZQ-resistant phenotype in *S. mansoni*. With the assays performed, it was demonstrated that the Pgp-like transporter *SmMDR2* has an involvement in PZQ-resistance in *S. mansoni* males, thus suggesting that MDR proteins are good targets to reverse the PZQ-resistant phenotype in *S. mansoni* males, deserving attention in future studies. Besides, the *ex vivo* assays performed showed that in the presence of an EPs inhibitor PZQ-susceptibility increased in males. Therefore, a combined therapy of PZQ and EPs inhibitors should be considered and studied in *in vivo* experiences since the administration of those inhibitors might increase worm susceptibility to PZQ.

Regarding *S. mansoni* females, it was not possible to observe a role for the Pgp-like transporter *SmMDR2* in PZQ-resistance. However, it was possible to determine that females are less susceptible to PZQ than males. Together these results strongly suggest that more than one pathway are involved in PZQ-resistance in *S. mansoni* worms. Therefore, it is of extreme importance to identify other mechanisms potentially involved in *S. mansoni* PZQ-resistance, in order to develop not only new drugs, but also new strategies for schistosomiasis control.

Adaptation of a technique previously applied to bacteria and cell cultures [5], allowed us to evaluate efflux pump activity in a multicellular organism. This is an important technical development that can be used in further studies to assess EPs activity in more complex parasites, specially, in a drug-resistance scenario.

A second section of this thesis aimed to compare the morphological characteristics of both PZQ-resistant and susceptible *S. mansoni* strains. With this analysis, it was possible to observe that when exposed to PZQ, resistant worms have less muscular contractions, less tegumental damage, higher viability and, after removal of the drug, a complete recover of motility. Those are important findings since any biological change can have repercussions in the pathology of the disease. This work makes it reasonable to hypothesize that the tegument of PZQ-resistant strain might have a different composition from susceptible one. It would be very interesting to explore this

difference, because knowing that the tegument can determine efficacy of the PZQ treatment, such studies could have great impact on schistosomiasis treatment [6-9].

Another important finding extracted from the morphological observation of the *S. mansoni* worms was the information that PZQ-resistant *S. mansoni* males and females are different, the latter being more resistant to PZQ treatment. Those findings reinforce the idea that the mechanisms involved in male and female PZQ-resistance may not always be the same, being necessary to study males and females in separate.

Besides the adult worms, we also analyzed the eggs of both strains, before and after PZQ exposure. This led to an important finding, the occurrence of differences in egg morphology between those strains. While PZQ-susceptible females do not produce eggs under PZQ-exposure, PZQ-resistant females do produce. However, the PZQ-resistant female eggs are smaller and have smaller spicules with different morphology (less saliente and less acute). These differences might have repercussions in what respects their tropism and it is even possibly that they reach the central nervous system, causing neuroschistosomiasis. If this hypothesis is correct, in regions where PZQ-resistant strain are circulating, changes in pathology and in disease transmission could occur. It is urgent to explore this hypothesis, determining not only whether differences in the tropism of PZQ-resistant eggs do occur, but also other possible differences occurring in this same life stage.

Proteomics is an important means to characterize species or strains [10], and in combination with classical biochemical purification methods, can provide information on expression and localization of proteins [11]. Here, we characterized for the first time the proteome of a PZQ-resistant *S. mansoni* strain and the respective isogenic susceptible strain, a very important step towards understanding PZQ-resistance. This allowed us to identify proteins possibly associated with PZQ-resistance. These are proteins that were found to be differentially expressed between the strains. Since these proteins may be related with the PZQ-resistance phenomenon their functional characterization should pursue in future studies aimed at identifying new drug targets for schistosomiasis control. The identification of the proteins putatively associated with PZQ-resistance in *S. mansoni* permits also to investigate the possibility of developing a diagnosis test to distinguish patients carrying PZQ-resistant strains from those with PZQ-susceptible *S. mansoni*. The development of such a test would constitute a major

step towards schistosomiasis control as it would render possible to adjust drug administration in order to increase treatment efficacy (perhaps even by combining PZQ with EPs inhibitors as suggested previously). Another advantage of being able to detect PZQ-resistant infections at an early phase is that it would make clinical staff more aware of the possible differences in disease pathology, preventing some complications (for example, the early detection of neuroschistosomiasis would facilitate treatment).

The proteome analysis made it also possible to identify proteins that were present only in females, being these good targets to identify the mechanisms underlying the decreased PZQ-susceptibility of females, when compared to males. Furthermore, some of these proteins may constitute targets, for schistosomiasis control. They should, therefore, be object of further analysis. In this context, the development and use of other techniques, such as genetic manipulation methods, will be crucial to further unravel the intriguing biology of schistosomes and their complex interaction with the host [11].

In summary, this thesis presents an innovative study, with important and relevant discoveries for public health. This work opens doors to other PZQ-resistance studies, and could possibly represent a basis to find a solution to the PZQ-resistance problem in a disease that affects millions of people worldwide.

2. References

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